5-2020

Nutritional Supplementation and Skeletal Muscle Health in Chronic Diseases

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Nutritional Supplementation and Skeletal Muscle Health in Chronic Diseases

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Food Science

by

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May 2020
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Abstract

Prevalence of chronic diseases in the United States keeps increasing in recent years. There are approximately four in ten adults having chronic diseases in America. Chronic diseases include obesity, aging, heart diseases, cancer and many others. This thesis mainly focuses on obesity and aging. Skeletal muscle performance is impaired in obesity and aging conditions. One common reason causing this could be higher fat mass and lower muscle mass. However, there is no study showing what pathways are involved in the imbalance of body composition. Therefore, the first objective of the thesis is to determine gene expression of selected pathways in the skeletal muscle from young and old, lean and overweight/obese participants. Leucine has been shown to activate protein translation initiation pathway mTORC1 in skeletal muscle. Further, mTORC1 is also known for regulating a variety of cellular pathways. Thus, the second objective of the thesis is to determine the effect and mechanisms of leucine supplementation on preventing intracellular lipid accumulation in skeletal muscle cells.

Acknowledgements

To my committee members, Dr. Jamie I. Baum, Dr. Sami Dridi, Dr. Yan Huang, Dr. Luke R. Howard, Dr. Sun-Ok Lee, and Dr. Walter G. Bottje, thank you for providing critical suggestions for my projects.

To my advisor, Dr. Jamie I. Baum, thank you for giving me huge support throughout my PhD career. My entire PhD career would not be successful without your guidance. Thank you for giving me opportunities to get involved in a lot of clinical trials so I can expand my expertise. You are the best mentor I have worked with.

For chapter 3 and 4, I would like to thank Dr. Robert R. Wolfe for his generous donation of muscle biopsy samples and valuable comments on manuscript drafting. I would also like to thank collaborators from South Korea, Dr. Il-Young Kim and Dr. Jiwoong Jang, for conducting hierarchical regression analysis.

For chapter 5, I would like to thank Dr. Martin Egan from Department of Plant Science for allowing and providing training for the confocal microscopy. To Dr. Sami Dridi, thank you for sharing your lab space and equipment. For Liz Greene, big thanks for the help with qPCR experiment.

To my families, thank you all for the support since I was born, I will always love you.

To my fiancé, thank you being at my side all the time.

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Wu H, Dridi S, Huang Y, and Baum JI. Leucine decreases intramyocellular lipid deposition in an mTORC1-independent manner in palmitate-treated C2C12 myotubes. American journal of physiology Endocrinology and metabolism 318: E152-E163, 2020.

1 Introduction

According to the report from Center for Disease and Control, there are six in ten adults in the US having one chronic disease and four in ten adults are having two or more chronic diseases (4). Chronic diseases such as obesity and aging are the leading cause for disability and death, and leading contributor for the annual health care cost of $3.5 trillion in the United States (4). Many factors contribute to the development of chronic diseases including smoking and exposure to second-handed smoke, poor nutrition including low vitamin, low protein, high sodium, and high saturated fats containing diets, sedentary life style, and excessive alcohol use (4). Prevalence of obesity and aging keeps rising in the United States. There are 15.2% of total population aged 65 or over, and there are 42.4% of total population obese in the United States (1, 13). Skeletal muscle taken from obese or aged adults shared some commons such as reduced relative muscle mass, increased fat infiltration, and increased fat accumulation surrounding muscle (5, 12). Muscle protein kinetics balance is correlated muscle growth, as muscle atrophy happens when muscle protein breakdown (MPB) is higher than muscle protein synthesis (MPS). Reduced basal MPS was reported in skeletal muscle from older or obese adults, compared to skeletal muscle from young or lean adults (10, 11). With the aspect of MPS, blunted activation of protein synthesis-related molecular pathway, the mechanistic target of rapamycin complex 1 pathway (mTORC1), was reported from skeletal muscle from diet-induced obese mice or aged mice (9, 18). With the aspect of MPB, there are three molecular pathways involved, autophagy, ubiquitin-proteasome system (UPS), and Ca^{2+}-calpain pathway. Autophagy and UPS are widely studied than Ca2+-calpain pathway. Especially in skeletal muscle, higher signal of autophagy and UPS are shown in the muscle from diet-induced mice or aged mice (6, 7). Fat accumulation could be the result of increased energy intake versus energy expenditure. As the center for ATP

production in cells, mitochondria has been shown reduced in total amount in skeletal muscle from obese or aging adults (5, 17). In addition, mitochondria is involved in fat oxidation as the rate-limiting enzyme, carnitine palmitoyltransferase 1 (Cpt1), localizes on the out membrane of mitochondria. Fat oxidation and Cpt1 enzymatic activity are shown reduced in muscle of obese adults (14). Further, increased gene expression related to fat synthesis is shown in muscle of aging adults (5). However, information on gene expression related to protein breakdown and energy metabolism are still lacking in skeletal muscle under obesity and aging conditions. Therefore, the first objective of this dissertation is to compare the gene expression of autophagy, UPS, mitochondria, and fat metabolism between young and old, or lean and obese, adults. It is hypothesized that muscle from older and obese adults had higher expression of autophagy, UPS, and fat synthesis-related genes; and lower expression of mitochondrial biogenesis and fat oxidation-related genes, compared to young and lean adults.

Nutrition such as leucine or branched-chain amino acids (BCAA)-enriched supplementation is a good option to improve skeletal muscle health in both obesity and aging conditions (16, 19). In addition, previous study from our lab showed BCAA-enriched high protein diet reduced adiposity in the skeletal muscle of obese Zucker rats (8). BCAA are different from other essential amino acids as the first step, conversion of BCAA to branched-chain keto-acids (BCKA) of their catabolism undergoes in skeletal muscle or central nervous system (15). Furthermore, leucine is different from valine and isoleucine as it can activate mTORC1 to improve translational initiation in skeletal muscle (2, 3). However, the effect of leucine supplementation on skeletal muscle health in obesity is still unclear. Therefore, the second objective of this dissertation is to determine the mechanisms and effect of leucine on

obese skeletal muscle. It is hypothesized that leucine supplementation could reduce lipid deposition in obese skeletal muscle through mTORC1 pathway.

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2 Chapter 1. Literature Review-Mechanisms Underlying Effects of Leucine on Obese Muscle

2.1 Abstract

Skeletal muscle health has been shown to be damaged under obesogenic conditions in several aspects such as decreased muscle mass, poor physical performance, and increased fat deposition. Leucine is well-known as the activator of the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which plays a central role in mediating protein turnover. Protein turnover is suggested to be directly linked to muscle mass. Therefore, the objective of the present review is to discuss current available evidence about the effect of leucine on improving skeletal muscle health under obesogenic conditions and underlying mechanisms. We conclude that as a nutritional intervention for obesity, leucine is able to increase protein synthesis, decrease protein breakdown, and improve mitochondrial function, and fatty acid catabolism. Regarding to current theory of elevated plasma level of leucine as a biomarker for obesity, we speculate the possible alterations in enzymes such as muscle specific BCATm and BCKDH would be responsible for elevated circulating leucine concentration observed in obese patients. Overall, although more and more studies have revealed the positive effects of leucine on skeletal muscle under obesogenic conditions, the exact mechanisms underlying the action of leucine is still unclear, and understanding of adipose-muscle tissue crosstalk would benefit for explaining high circulating BCAAs level in obese patients.

2.2 Introduction

According to the Centers for Disease Control and Prevention (CDC), 42.4% of US adults are obese (39). Moreover, chronic diseases such as diabetes, cardiovascular diseases, stroke, non-

alcoholic fatty liver disease (NAFLD) and certain types of cancer are also associated with obesity (19). Physiologically, skeletal muscle from obese patients is impaired in many aspects such as structure (muscle fiber composition) and muscle strength (contractile force) (27, 78-80). Muscle impairment in obesity is also associated with poor quality of life, decreased motility, self-care, usual activities and pain/discomfort (15). The health complications associated with obesity place a heavy economic burden on governments around the world (29). It is estimated that $149.4 billion is invested annually for prevention and treatment of obesity in the United States (51). In most cases, obesity is caused by over-nutrition. Over-nutrition results in over-accumulation of adipose tissue such as subcutaneous fat and visceral fat, which causes imbalance in body composition, in which higher fat mass and lower muscle mass found in obese patients (70). A better understanding of the molecular pathways dysregulated under obesity conditions will benefit for further designing of intervention to reduce weight. Currently, several treatments targeting weight loss such as exercise (2) and healthy diets (31) have been shown to improve muscle health. For example, leucine-enriched high protein diet increased muscle protein synthesis regulator activity, mechanistic target of rapamycin complex 1 (mTORC1), in obese Zucker rats (30). Leucine, one of branched-chain amino acids (BCAAs), bypasses portal venous system to undergo the first and reversible step of catabolism in before entering the circulating system. The enzyme catalyzes this step is mitochondrial form of branched-chain amino acid transaminase (BCATm),which highly expresses in skeletal muscle, and cytosolic form of BCAT (BCATc), which highly expresses in brain and peripheral nerves. This feature makes leucine metabolism unique among amino acids. Leucine functions not only as a building block for protein synthesis and a source for ATP production, but also as an activator of mTORC1, and regulator for other cellular metabolism such as autophagy, mitochondrial biogenesis,

adipogenesis, lipid metabolism and so on (69). Impairments in metabolic pathways such as mTORC1 (88), autophagy (28), mitochondrial function (50), and fatty acid metabolism (49) have been reported in the skeletal muscle of individuals with obesity and animal models of diet-induced obesity. In recent years, research has focused on the effect of leucine supplementation on skeletal muscle under obesogenic conditions using animal models and identifying potential mechanisms of action for leucine's role in obesity treatment and or prevention (6, 9, 32, 55, 59, 65, 96). However, the detailed mechanisms involved in the effect of leucine on improving muscle functions in obesity conditions is less discussed. Thus, the present review is aimed to summarize and discuss current evidence regarding mechanisms involved in skeletal muscle impairment under obesogenic conditions and the role of leucine supplementation in improving muscle function. In addition, this review aims to provide possible new directions for future studies.

2.3 Mechanisms underlying skeletal muscle impairments in obesity

2.3.1 Imbalanced protein turnover in obese muscle

Skeletal muscle is constantly synthesizing and degrading proteins. The difference between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) is defined as net balance (NB). Deficits in MPS and/or increase in MPS, which results in a negative net balance, will cause loss of skeletal muscle protein. Conversely, accretion of skeletal muscle protein can be a result of rise in MPS and/or reduction in MPB. Muscle mass is shown to be correlated with muscle protein turnover. For example, it is shown that higher dietary protein intake is associated with higher skeletal muscle mass (77). In addition, a significant reduction in MPS in human muscle from leg disuse (33). Later studies also confirmed reduced MPS in human undergoing 28

days of bed rest (68). In contrast to MPS, measurement of MPB is still a technical challenge since the current available methods, endogenous labeling of ^{2}H-alanine by $^{2}H_2O$ consumption (44) and L-ring-$^{13}C_6$-phenylalanine infusion (81), measuring MPB through indirectly calculating the disappearance rate of labeled alanine or phenylalanine. Recent study compares these two methods and reports failure in MPB measurements (43). In addition, these researchers consider labeling essential amino acids tracer is inappropriate as possible significant and prolonged labeled amino acid recycling (43). A novel method using labeled methyl-D_3-^{13}C-methionine is shown to measure MPB in C2C12 myotubes, but this method still needs further verification in human muscle (87). Thus, measurement of MPB-related pathways such as autophagy is suggested to be an add-on to tracer methods.

Increased MPB in the post-absorptive state has been reported in young healthy obese men, and MPS in obese muscle is increased to a lower level than lean muscle in post-absorptive state (38). A similar reduction in MPS response under fed conditions was reported in obese elderly (64). Rate of labeled phenylalanine appearance, an indicator for MPS, is reported to be lower in skeletal muscle from obese young adults compared with lean young adults (5). Increased MPB is shown to be correlated with moderate upper-body obesity (47). This has also confirmed in animal studies (73). For example, decreased 24h MPS was demonstrated in gastrocnemius muscle from obese Zucker rats (66). In addition, increased relative ratio of urinary muscle derived 3-methylated-histidine to creatine, which reflects degradation of myofibrillar proteins as described in previous paragraph, is shown in muscle of obese Zucker rats (73). These results suggest that a higher ratio of MPB to lower MPS exists in the skeletal muscle of obese adults.

2.3.2 Blunted mTORC1 response in obese muscle

The mammalian Target of Rapamycin Complex 1 (mTORC1) is a well-established pathway for regulating protein translation initiation in skeletal muscle (37). The mTORC1 are shown simultaneously dysregulated in skeletal muscle under obesogenic conditions (8, 66, 89). There are two direct downstream proteins of mTORC1, S6 Kinase 1 (S6K1) and eukaryotic translation initiation factor 4E binding protein (4EBP1), known as indicators of mTORC1 activity (26, 90). Relative content of phosphorylated S6K1 and 4EBP1 are reduced in vastus lateralis muscle from obese patients (88). Blunted activation of mTORC1 to feeding and exercise in vastus lateralis muscle has also reported in obese patients (7). In addition, in genetic obese animal model, it has been demonstrated that decreased phosphorylation of S6K1 in the skeletal muscle (66). Mice fed a high-fat diet showed decreased phosphorylation level of S6, target of S6K1, in muscle, and this is exacerbated by addition of rapamycin (61). Collectively, these results suggest a blunted mTORC1 response to MPS-improving methods under obesogenic conditions (Figure 2.1A).

2.3.3 Dysfunctional skeletal muscle mitochondria in obesity

Mitochondria are responsible for intracellular energy metabolism in skeletal muscle as the center for ATP production. All energy sources including carbohydrates, fat, and proteins finally enter the tricarboxylic cycle (TCA) and electron transport chain (ETC) in mitochondria for energy production in the form of ATP in muscle. Mitochondria sense intramuscular energy status in the form of the ratio of ATP to AMP (86). Therefore, activity of enzymes in the ETC directly determines ATP production in skeletal muscle. In research comparing mitochondrial ETC in skeletal muscle taken from lean and obese individuals, complex I of ETC that initiates the electron transport, NADH: O2 oxidoreductase was showed less active in skeletal muscle from obese adults. It is reported that higher production rate of pyruvate and palmitoyl-DL-carnitine

reactive oxygen species (ROS) from mitochondrial ETC in the skeletal muscle from obese individuals, compared to lean counterparts (53). This suggests a negative status of maintaining extra-mitochondrial ATP free energy by a low metabolic flux. Moreover, in the same paper, relative lower abundance of electron transfer chain complex I and enzymes related to BCAA metabolism were found in obese people (53). In addition, animal study reported that mice, develops obesity after 16 weeks of high-fat, high-sucrose diet feeding, showed lower ratio of mitochondrial DNA content versus nuclear DNA content, accompanied with decrease enzymatic activity of electron transport chain and mitochondria morphology, compared to its counterparts (12). Taken together, these results shows the activity of mitochondrial ETC is dysfunctional under obesity conditions in skeletal muscle (Figure 2.1B).

Uncoupling proteins that are capable of uncoupling the proton gradient produced by ETC for ATP production, especially uncoupling protein 3 (UCP3), was firstly discovered in human skeletal muscle in 1997 (84). Inconsistent results of skeletal muscle UCP3 expression are reported between human and animal studies in obesity conditions. Two human studies showed no difference in UCP3 expression between lean and obese adults (52, 83). However, diet-induced obese mice showed decreased expression of UCP3 in skeletal muscle compared with normal diet-fed mice (25). After that, another study demonstrated transgenic mice that overexpressing human UCP3 had lower weight comparing with obese littermates even had higher food intake (18). In addition, mice overexpressing UCP3 showed improved glucose clearance and lower fasting plasma glucose and insulin level, which indicated enhanced glucose sensitivity (17). Similar research demonstrated *in vivo* overexpression of UCP3 in skeletal muscle protected mice being obese and insulin resistant from high-fat-diet feeding (17). The discrepancy in the expression of skeletal muscle UCP3 between human studies and animal studies could be

explained by species difference. Therefore, further study on comparing UCP3 expression and protein content in skeletal muscle between lean and obese adults is needed (Figure 2.1B).

2.3.4 Autophagy & ubiquitin proteasome system

Autophagy is activated when misfolded proteins or damaged organelles accumulate intracellularly (54). During the process, proteins and/or organelles are first engulfed in autophagosome that then fuses with lysosomes to degrade the engulfed material for the purpose of energy recycle for other cell metabolism-related pathways. Therefore, autophagy plays a key role in protein turnover, especially protein breakdown. Reports on the changes in autophagy in skeletal muscle taken from obese conditions are inconsistent. It is reported that in female rats fed with high-fat diet for 16 weeks, there is no alternation in the indicators of autophagy such as microtubule-associated protein 1A/1B-light chain3 (LC3) lipidation and protein content of Beclin1 and Atg7 in the soleus and plantaris muscle, accompanied with no alternation in lysosomal enzyme activity (16). Our previous *in vitro* study also demonstrates no alternation in protein content of p62 and lipidated LC3 in palmitate-treated C2C12 myotubes (91). However, another study using primary human skeletal muscle cells from lean and obese people, showed slower autophagic/lysosomal flux and lower expression of autophagy-related genes such as autophagy-related gene 4B (ATG4B) and gamma amino butyric acid receptor associated protein (GABARAPL-1) in skeletal muscle cells from obese patients (11). Similarly, Fan and Xiao showed higher autophagy flux indicated by higher LC3 lipidation and autophagosome formation in diet-induced obese mice (28). This could be explained by different models used, primary cell culture versus animal model. Taking these results together, it is apparent that autophagy is dysregulated in skeletal muscle of obesogenic conditions (Figure 2.1C). However, more

investigation is needed for better understanding how autophagy alters in skeletal muscle of obese patients.

Ubiquitin proteasome system (UPS) is another major intracellular mechanism for protein breakdown. Briefly, the whole process includes five steps: 1) Attachment of ubiquitin to ubiquitin activating enzyme (E1); 2) Transfer of ubiquitin to ubiquitin conjugase (E2); 3) conjugation of ubiquitin within substrate through ubiquitin ligase (E3); 4) formation of polyubiquinated substrate; 5) degradation of substrate by 26S proteasome and release of ubiquitin. In skeletal muscle, there are two muscle-specific E3 ligases are identified, Atrogin1 (35) and MuRF1 (10). Skeletal muscle cells taken from obese patients have higher proteasome enzyme activity under starving condition than lean counterparts, and higher expression of FOXO3, a transcription factor for Atrogin1 and MuRF1 (11) (Figure 2.1C). In the skeletal muscle of mice fed with high-fat diet, expressions of Atrogin1 and MuRF1 are higher than mice fed with control diet (1). However, there is still a lack of research in how UPS is dysregulated under obesity conditions.

2.3.5 Fat Metabolism

In skeletal muscle, fat deposition can be divided into two categories: extramyocellular lipid (EMCL) and intramyocellular lipid (IMCL). EMCL and IMCL, measured by nuclear magnetic resonance (NMR) spectroscopy, are higher in the tibialis anterioris muscle of overweight and obese men, and are positively correlated with abdominal fat (24). Another study also showed increased IMCL and EMCL in overweight and obese men, and an increase in IMCL-to-creatine ratio as BMI increases (82). The cause for this could be dysregulations in both fatty acid oxidation and fatty acid synthesis.

Normal weight men that were fed a high-fat diet for 5 days showed lower fatty acid oxidation rate and lower fatty acid suppressibility of pyruvate oxidation in the skeletal muscle, with an increased in serum free fatty acid acids (FFAs) (13). This study suggests that the elevated level of serum FFAs might correlated with lower fatty acid oxidation. In addition, total lipid catabolism is shown decreased in skeletal muscle cells taken from obese patients (49). Consistent with this finding, it is shown that a trend of decrease found in the expression of carnitine palmitoyltransferase 1 (Cpt1), the rate-limiting step in the transport of fatty acids into mitochondria for oxidation, in the skeletal muscle of female Cynomolgus Macaques fed with Western diet (36). In mice, it is reported that phosphorylation acetyl-CoA carboxylase (ACC) by AMP kinase (AMPK) is necessary for fatty acid oxidation in the skeletal muscle (67). ACC is involved in the synthesis of short chain fatty acids, and is inhibited by its phosphorylation. It is confirmed by *in vitro* study that phosphorylation of ACC is decreased in palmitate-treated C2C12 myotubes (76). These reports suggests a potential higher fatty acid synthesis in the skeletal muscle under obesogenic conditions. In genetic obese animal model, *ob/ob* mice, there is higher content of long chain fatty acids such as 16:0, 18:1, and 18:0 triacylglycerol, and higher expression of fatty acid elongase in the skeletal muscle (41). Previous animal studies from our lab also demonstrated higher gene expression of fatty acid synthase (FAS) in skeletal muscle of obese Zucker rats comparing with lean controls (30). Overall, higher fatty acid synthesis and lower fatty acid oxidation signals are possible factors contributing to the development of both ECML and IMCL (Figure 2.1D).

2.4 The effect of leucine on different pathways in obese muscle

Healthy diets benefits obese patients in multiple aspects. For example, consuming a high protein diet for 12 weeks in overweight and obese women showed a significant reduction in body weight, in which fat mass was reduced and lean mass was maintained (63). In addition to weight loss and reduced adiposity, obese patients who consumed a high protein diet for 12 weeks showed improved profile of cardiovascular diseases with elevated triacylglycerol (20). High protein supplementation also benefits weight loss and lean mass preservation in obese patients (31). High protein diet or high protein supplementation contains substantial amount of branched-chain amino acids (BCAA), valine, isoleucine, and leucine. Among valine, isoleucine, and leucine, leucine has been shown to activate mTORC1 pathway to increase protein translational initiation in muscle (3). Therefore, it is interesting to understand whether dietary leucine supplementation would improve obesity and muscle function. The sections below summarize current evidence on the mechanisms involved in the effect of leucine supplementation on obese muscle.

2.4.1 Leucine improves obesity through activating mTORC1 signaling in muscle

Leucine upregulates protein synthesis in muscle through promoting mTORC1 signaling. Previously, the effect of leucine supplementation on improving muscle function has been reported in cell studies (48), animal studies (22) and human studies (31). Therefore, researchers started to hypothesize whether leucine can improve obesity-related skeletal muscle dysfunctions through mTORC1 signaling. Dietary supplementation of leucine in drinking water for mice fed with high-fat diet showed reverse effect on metabolic abnormalities such as glucose tolerance, hepatic steatosis, adipose tissue inflammation. Moreover, leucine caused upregulation in mTORC1 signaling, indicated by increased phosphorylation of S6K1, the direct downstream

molecule of mTORC1(62). Another study showed that chronic leucine supplementation improves insulin sensitivity and reduced oxidative stress, accompanied by stimulation in mTORC1 signaling in skeletal muscle (58). In addition, central administration of leucine in hypothalamus stimulates mTORC1 signaling and decreases food intake and body weight in rats (21). In human studies, whey protein supplement enriched in leucine appears to increase fat mass loss and prevent more lean muscle mass loss in obese people, compared to normal weight counterparts (31). Notably, sestrin2 was shown to be a sensor for leucine to activate mTORC1 intracellularly (90). However, in human muscle, sestrin2 was not hyper-phosphorylated by ingestion of a milk protein concentrate (95). Another study showed leucyl-tRNA synthetase is an intracellular leucine sensor for mTORC1 activation (40). To our knowledge, the only evidence for the role of leucyl-tRNA synthetase in muscle is its involvement in C2C12 myoblast differentiation (71). Thus, it is unclear what the intracellular leucine sensor in muscle is. The role of mTORC1 signaling in promoting protein synthesis rate in skeletal muscle has been emphasized (Figure 2.2A). However, there is still a deficit in the amount of investigation about the effect of leucine supplementation or protein supplementation that high in leucine on upregulating protein synthesis through mTORC1 signaling in obese patients.

2.4.2 Leucine improves obesity through mitochondria in muscle

In obesogenic conditions, especially in skeletal muscle, an organism heavily dependent on oxidative phosphorylation, mitochondrial dysfunctions are common (50). The positive effect of leucine supplementation on mitochondrial function has been reported in cell (72) and animals (46). In mouse myotubes cell model, C2C12, 24h leucine treatment led to increased mitochondrial content, which is related to upregulated mitochondrial biogenesis genes, PPARβ/δ, expression (72). For simulating obesogenic conditions *in vivo*, palmitic acid has been introduced

to induce obesity-linked dysfunctions *in vitro* (91). Previous study from our lab also showed increased mitochondrial contents in leucine-supplemented palmitate-treated C2C12 myotubes (91). In addition, mitochondrial function-related genes (e.g. nuclear respiratory factor 1 (NRF1) and ATP synthase) are upregulated by dietary leucine supplementation in piglets (46). Based on the protective effect of leucine supplementation on mitochondrial function, biogenesis and gene expression, researchers considered whether the positive effect caused by leucine supplementation in obesity is related to improved mitochondrial function. In high-fat diet-induced obese mice, leucine supplementation reduces fat mass, which is correlated with upregulation of mitochondrial metabolism gene, PGC1α (56). Another study showed that leucine supplementation improve mitochondrial biogenesis, revealed by enhanced mitochondrial DNA copy number, increased protein content of oxidative phosphorylation complex I to IV, augmentation in serum ATP content at the stage of hyperglycemia (60). More recently, leucine supplementation increases intermyofibrillar, not subsarcolemmal, mitochondrial size in diet-induced obese mice (14). Conclusively, leucine has been shown to improve mitochondrial function, quality, and number in skeletal muscle that under obesogenic conditions (Figure 2.2B).

2.4.3 Leucine improves obesity through protein breakdown-related signaling in muscle

The interaction between leucine and autophagy has been reported in human embryonic kidney 293 (HEK293T) cells (92). Increased signal of autophagy, present by higher LC3 lipidation and p62 protein content, is observed in leucine-deprived cells (92). In addition, leucine supplementation reduces Barkor puncta formation, an indicator for autophagosome formation, *in vivo* (92). Low-protein diet-induced elevation in triglyceride accumulation, a common phenomenon happens during the development of obesity, was shown restored by leucine supplementation. Simultaneously, level of autophagy, revealed by protein content of LC3-II, was

reduced in leucine supplemented low-protein diet-fed group (93). The low-protein diet used in this study does not completely simulate diet-induced obesity. High-fat diet or Western diet is suggested to be used for further investigation on the mechanism of leucine supplementation on autophagy in skeletal muscle.

The anti-atrophy effect of leucine on skeletal muscle through prevention of expression of MuRF1 and Atrogin-1, which play essential role in skeletal muscle atrophy, was shown by Baptista et al (4). Furthermore, Rasmussen et al reported higher muscle fractional synthesis rate in a high leucine consumption (3.5g leucine) group of young men and women comparing with a typical high quality protein consumption (1.8g leucine) group of young men and women, although no changes were detected in ubiquitin-proteasome system between two groups (34). However, the supplementation used in this study is high quality protein, not single leucine supplementation, which the effect of leucine could be blunted by other amino acids. It is still of interest in investigating the effect of leucine supplementation on protein degradation pathways such as autophagy and UPS in skeletal muscle under diet-induced obesity conditions. A schematic diagram of the action of leucine on autophagy and UPS in skeletal muscle under obesity condition can be found in Figure 2.2C.

2.4.4 Leucine improves obesity through fat metabolism in muscle

Leucine supplementation is shown to increase phosphorylation level of ACC in skeletal muscle of high-fat diet fed mice (55). This indicates leucine supplementation might be able to block fatty acid synthesis. In addition, overweight and obese people consuming a dietary supplementation containing 750 mg leucine showed increased fat oxidation rate (94). Consistent with these results, *in vitro* study from our lab also showed leucine reduces IMCL and upregulates Cpt1 expression in palmitate-treated C2C12 myotubes (91). However, there is also inconsistent

results showing branched-chain amino acid (BCAA) restriction increases fatty acid oxidation in skeletal muscle of obese Zucker rats (85). This could be explained by the difference in the experimental models used. Obese Zucker rats has a mutated leptin receptor leading to hyperphagia, which is different mechanistically with high-fat diet-induced obesity. Summarization of the effect leucine on fat metabolism in skeletal muscle under obesity condition is shown in Figure 2.2D. Apparently, there are two major problems needed to be addressed in the future: 1) impairments in leucine catabolism in skeletal muscle under obesogenic conditions; 2) the effect and mechanism of leucine on the fat metabolism in skeletal muscle under obesogenic conditions.

2.5 Conclusion

The mechanism underlying the effect of leucine on skeletal muscle under obese circumstance is present in Figure 1.2. Leucine, as a well-known activator of mTORC1 pathway, has been shown to improve obesity-related phenotypes (31, 57). In addition, leucine supplementation is shown to promote mitochondrial biogenesis (45) and mitochondrial size (59) in skeletal muscle of obese models. As a result, increased ETC enzymes activity (59) and ATP production (45) are observed. Autophagy signal, present by LC3 lipidation, is also decreased by leucine supplementation in skeletal muscle, which suggests a decrease in protein degradation (93). Lastly, leucine supplementation is present to decrease fatty acid synthesis through inhibiting ACC activity (55) and increase fatty acid oxidation through upregulating Cpt1 expression (91) in skeletal muscle of obese models.

Elevation of plasma BCAAs is commonly seen in obese models, and studies have suggested possible alternations in the two enzymes for catalyzing the first two steps of BCAAs catabolism,

mitochondrial branched chain amino acid aminotransferase (BCATm) and branched chain α-keto acid dehydrogenase (BCKDH, E1α subunit) complex, in the omental and subcutaneous fat tissues, not liver or muscle, may contribute to the rise in plasma BCAAs (75). Same group of researchers show that by knocking out BCATm globally, mice fed with high-fat diet are significantly resistant to obesity (74). In addition, adipose tissue, not muscle, is suggested to regulate circulating BCAAs level in *in vivo* (42). Recently, one study using diet-induced insulin resistant animal model shows impaired skeletal muscle BCATm activity, while adipose tissue BCATm remains unchanged (23). Moreover, they find higher phosphorylation of BCKDH only in skeletal muscle of insulin resistant animals (23). Therefore, we suggest more studies related to the mechanisms of adipose tissue-skeletal muscle crosstalk with leucine supplementation under obesogenic conditions should be carried out in the future.

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2.7 Figures

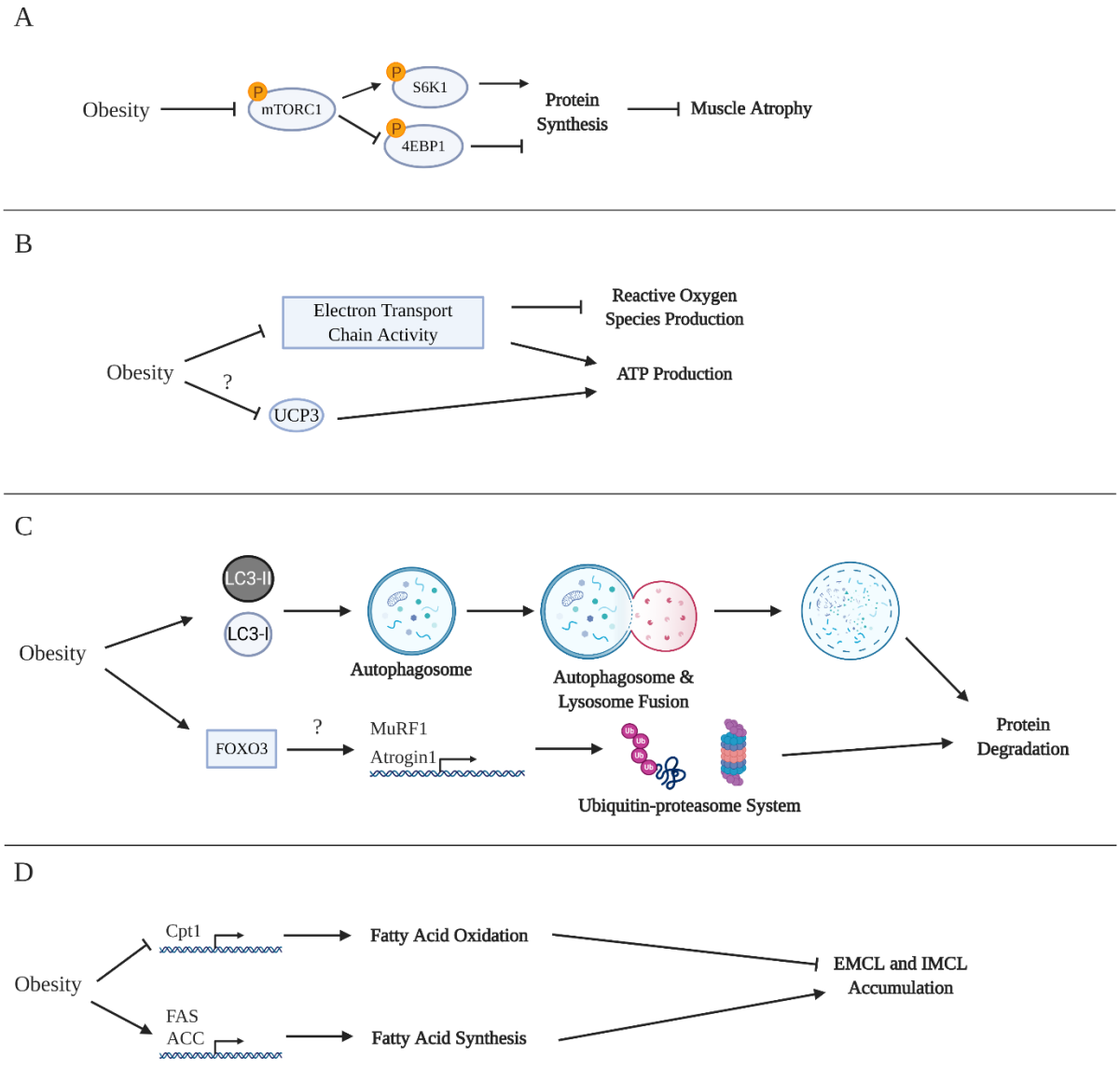

Figure 2.1. Schematic pathways dysregulated by obesity in skeletal muscle. Summary of current evidence on dysregulated pathways related to muscle protein turnover and energy metabolism in obesity condition. (A) Decreased muscle protein synthesis rate could be a result of blunted mTORC1 activation in obesity condition; (B) Obesity decreases electron transport chain activity, and UCP3 expression possibly, to cause decreased ATP production and accumulated reactive oxygen species in skeletal muscle; (C) Obesity increases protein degradation through LC3 lipidation-mediated autophagy and FOXO3-mediated ubiquitin proteasome system in skeletal muscle; (D) Obesity increases extramyocellular and intramyocellular lipids through decreasing Cpt1-regulated fatty acid oxidation and ACC and FAS-regulated fatty acid synthesis. Figure drawn using Biorender app, https://biorender.com/.

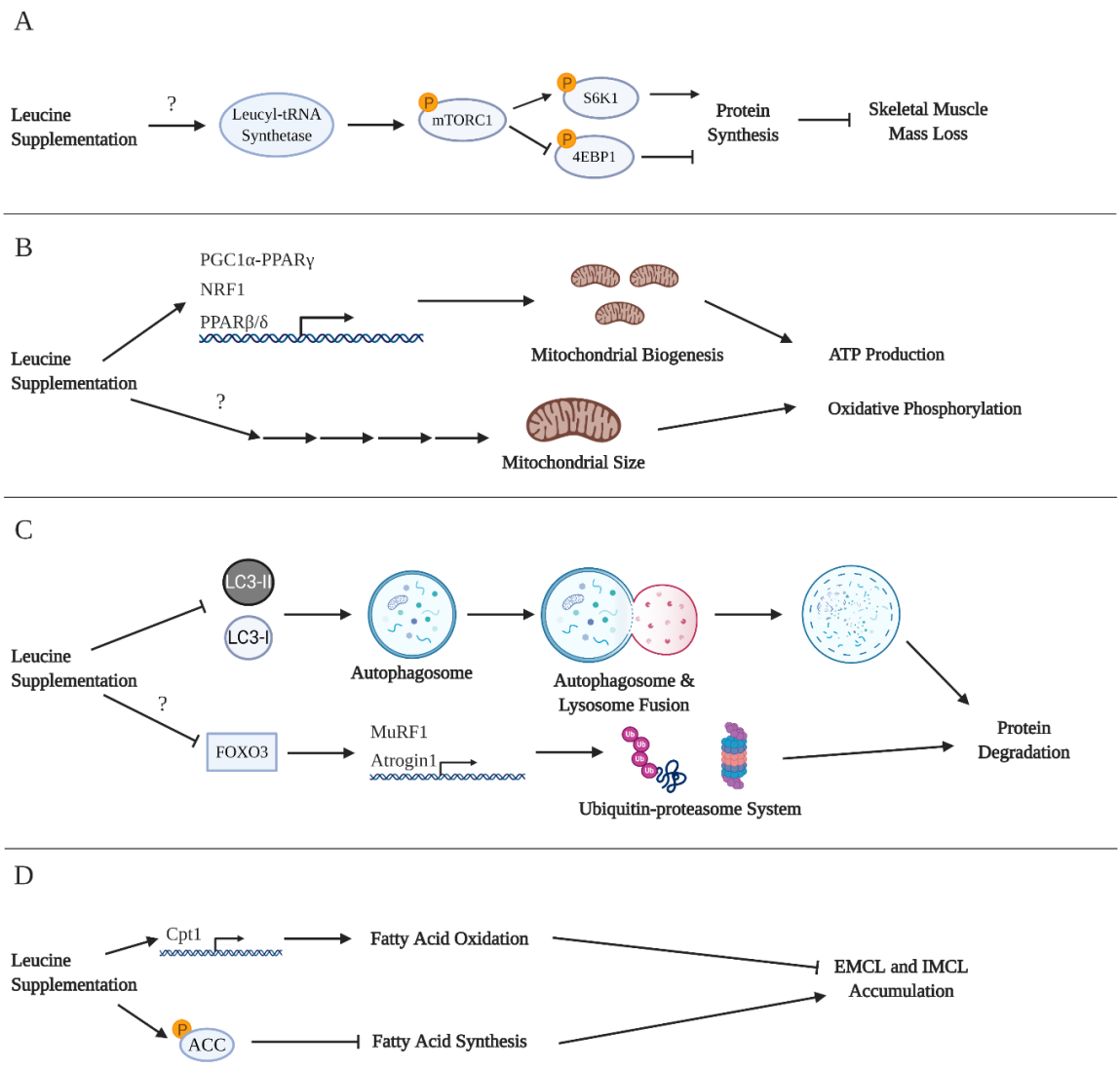

Figure 2.2. Possible mechanisms underlying the effect of leucine supplementation for improving skeletal muscle in obesity. This scheme encapsulates current evidence on possible mechanism involved in effect of leucine supplementation in skeletal muscle and subsequent physiological consequences. (A) Leucine activates mTORC1 pathway to promote protein synthesis for prevention of muscle loss in obesity, while the role of leucine sensor, leucyl-tRNA synthetase, is unclear; (B) Leucine promotes mitochondria production through increasing expression of mitochondrial biogenesis-relate genes such as PGC1α, PPARγ, NRF1, PPARβ/δ, and increases mitochondrial size through unknown pathway, to increase ATP production in obese muscle; (C) Leucine reduces LC3 lipidation to decrease autophagy signal, and reduces ubiquitin-proteasome system possibly through inhibiting Atrogin1 and MuRF1 expression, to downregulate protein degradation in obese muscle; (D) Leucine decreases intramyocellular lipid and extramyocellular lipid through upregulating carnitine palmitoyltransferase 1-mediated fatty acid oxidation, and through inhibiting acetyl-CoA carboxylase-mediated fatty acid synthesis. Figure is drawn by Biorender app, https://biorender.com/.

3 Chapter 2. Literature Review-Mechanism Underlying Effects of Leucine on Aging Muscle

3.1 Abstract

Age-related sarcopenia continues to exacerbate life quality of elderly in the United States. Continuous loss of skeletal muscle mass leads to higher risk of disability and death in elderly. This could be imbalances in muscle protein kinetics and energy metabolism. Thus, the first objective of this review was to summarize current evidence showing dysregulated molecular pathways involved in muscle protein kinetics and energy metabolism in aging conditions. Leucine is well-known as the activator of the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which plays a central role in mediating muscle protein synthesis. Therefore, the second objective of this review was to summarize current evidence showing the effects of leucine on molecular pathways related to muscle protein kinetics and energy metabolism in aging conditions.

3.2 Introduction

In 2015, 9 percent of total world population, 617 million were aged 65 and older. It was estimated that the older population will be approximately 1 billion by 2030, and 1.6 billion by 2050 (47). Older adults suffer continuous skeletal muscle loss, also known as age-related sarcopenia. The most direct consequence of skeletal muscle loss is falling down that leads to other injuries; e.g. bone breakage. It is shown that one out of four older adults falls each year in the United States (38). Falls can cause non-fatal injuries such as hip fractures, cataclasis, head trauma, and would lead to death in older adults (3). It cost the whole nation $50 billion a year to treat older adults with injuries from falls (15). In addition, it was predicted that the cost for

treating falls in the older adults would be $101 billion in 2030 if the rates of falls are not reduced (19).

There are two factors contribute to poor physical performance in the older adults: skeletal muscle loss and dysregulated fat deposition. Further, skeletal muscle loss is suggested to be caused by higher muscle protein degradation rate and lower muscle protein synthesis rate (46, 48). Dysregulated fat deposition can be the results of increased fat infiltration (45) and fat accumulation (14). There are a variety of components in different pathways identified to be involved in age-related sarcopenia and fat deposition dysregulation. Therefore, the first objective of the present review is to summarize current evidence in determining the role of multiple pathways in the development of sarcopenia and higher fat deposition in the skeletal muscle of older adults.

Leucine has been shown to activate the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which is the main regulator for protein synthesis in the skeletal muscle (1). Activation of mTORC1 pathway was also shown to inhibit protein degradation-related pathways such as autophagy (55) and ubiquitin proteasome system (UPS) (27) in muscle. Thus, there are substantial studies have been done to reveal the effect of leucine supplementation on autophagy and UPS in aging muscle. However, the effect of leucine supplementation on fat deposition and related mechanism in aging muscle is still unclear. Therefore, the second objective of the present review is to summarize mechanisms including mTORC1, autophagy, UPS, and fatty acid metabolism, underlying the effect of leucine on aging muscle.

3.3 Dysregulated pathways underlying aging muscle

3.3.1 mTORC1

The mTORC1 pathway was shown to be less activated in the skeletal muscle of older adults, as revealed by lower relative phosphorylation level of mTOR, S6 kinase 1 (S6K1), and eukaryotic initiation factor 4E binding protein 1 (4EBP1), than young counterparts after 6 h of exercise (17). In addition, muscle fractional synthesis rate was shown to increase to a lower extent in the muscle of older adults (17). Similar impairments in the relative activation of mTORC1 pathway after a bout of resistant exercise were reported in the muscle of older adults (16). It is interesting to point out that the relative phosphorylation of mTORC1 pathway were not different in the skeletal muscle from young and old adults in these two studies (16, 17). However, in another recent animal study, it is shown that relative phosphorylation of mTORC1 pathway at baseline was higher in the skeletal muscle (11). The discrepancy could be explained by the different muscle samples used. In the animal study, soleus muscle, a typical type I fiber, was used. While in the two human studies, vastus lateralis, was used. Vastus lateralis muscle in human is consist of type I and type II fiber, and the number and size of type II fiber was shown to decline in the older adults (6). However, another study showed no difference in muscle fractional synthesis rate between young and old adults, and higher absolute phosphorylation levels of mTORC1 pathway in the vastus lateralis muscle of older adults (25). Similar to this result, absolute phosphorylation level of S6K1, not mTORC1, was observed in the vastus lateralis muscle from older adults (37). Notably, in these two study, they only measured the absolute phosphorylation level of mTORC1 and its components, which did not reflect the true relative activation level of mTORC1 pathway.

3.3.2 Autophagy

Autophagy is a cellular process in which damaged cellular components such as misfolded protein and organelles are transported to lysosomes for degradation. Autophagy can be categorized into three kinds: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (CMA). The discussion below will focus on macro-autophagy. Briefly, autophagy has three stages: 1) Formation of autophagosome; 2) Fusion of autophagosome; 3) Degradation of the materials in lysosome. Therefore, autophagy has been suggested to facilitate protein degradation in aging muscle. For examples, expression of Beclin1, a protein involved in initiation of autophagosome formation, and p62, a protein involved in autophagosome formation, were found upregulated in the vastus lateralis muscle of older adults (53). In human studies, higher protein content of Beclin1 was found in the plantaris muscle of aged rats (50). However, protein content of autophagy-related gene 7 (ATG7) and autophagy-related gene (ATG9), which are involved in autophagosome formation, and microtubule-associated protein 1 light chain 3 (LC3) lipidation, which is an autophagosome marker, were found unchanged in the muscle of aged rats (50). Data from our lab found higher expression of p62, ATG7, and Beclin1 in the vastus lateralis muscle of older adults (unpublished data). However, in animal studies, the results are inconsistent. Higher protein content of p62 and lower LC3 lipidation were reported in the muscle of aged (27-month-old) mice (56). Meanwhile, protein content of ATG5-ATG12 conjugate, which is necessary for LC3 lipidation, was found lower in the aged murine muscle (56). Another animal study used young (3-month-old) and aged (24-month-old) mice showed higher accumulation of cytosol Beclin1 and p62 in the quadriceps muscle of aged mice, while content of Beclin1 and p62 in nucleus and membrane were unchanged (34). Overall, the discrepancies in the autophagy between human studies and animal studies could be the

consequence of species characteristics. Furthermore, there could be a balance in the flux of autophagy, as proper activation of autophagy was shown to be necessary for maintaining muscle mass (26). Thus, understanding of the balance between proper-activation and over-activation of autophagy is needed for future study in aging muscle.

3.3.3 Mitochondria

Mitochondria is the powerhouse that produce ATP for all cellular process. In muscle, ATP generated by mitochondria enable muscle to contract and move. Mitochondrial capacity or functio are reported in turns of numbers, sizes, electron transport chain (ETC) complexes enzymatic activity, and so on. Muscle taken from human undergoing exercise training had multiple improvements in mitochondria such as bigger mitochondrial size (29). In addition, muscle-specific knockout of mitofusins, which are important for mitochondria fusion, in mice significantly decreased muscle performance (7). In aging adults, there were lower mitochondrial ATP production and lower mitochondrial DNA copy number in the skeletal muscle taken from older adults (22). In addition, mitochondrial bioenergetics revealed by cytochrome c oxidase (COX) enzymatic activity was shown in the skeletal muscle of older adults, accompanied with lower protein content of mitochondrial biogenesis markers, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) (21). Changes in mitochondrial morphologies such as lower mitochondrial number and decreased mitochondrial size revealed in the subsarcolemmal component were observed in the skeletal muscle of older adults (12). Mitochondrial dysfunction was suggested to contribute to the programmed cell death of skeletal muscle in aged mice, as necrosis was found to be predominant in atrophic and morphologically broken ETC-disrupted muscle fibers (10). Overall, mitochondria in aging skeletal muscle has

been shown to be abnormal in multiple aspects, and the abnormalities further contribute to the death of muscle fibers to exacerbate poor muscle performance.

3.3.4 Fat Metabolism

Significant increased fat deposition, especially intermuscular fat infiltration, was found in the gastrocnemius medialis and soleus muscle of older adults (13). This could result from decreased fatty acid oxidation (44) and increased fatty acid synthesis (43) in aging muscle. On the side of fatty acid oxidation regulation, there are two major enzymes, carnitine palmitoyltransferase I (Cpt1) for β-oxidation of long chain fatty acids (LCFAs), which is localized at the outer membrane of mitochondria; and Cpt2 for β-oxidation of medium chain fatty acids (MCFAs), which is localized at the inner membrane of mitochondria. Skeletal muscle specific isoform of Cpt1, Cpt1b, knockout in middle-aged mice showed impaired LCFAs oxidation and increased intramyocellular lipid (IMCL) accumulation (49). Primary myotubes taken from Cpt2 deficient patients showed higher content of C16:0 acylcarnitines and lower fatty acid oxidation flux (8). However, there is no direct evidence showing deficiencies of Cpt1 or Cpt2 in aging muscle. On the side of fatty acid synthesis, activity of acetyl-CoA carboxylase (ACC), which catalyzes the step to provide malonyl-CoA for fatty acid synthesis, was found increased in vastus lateralis muscle of older adults (23).

3.4 Pathways involved in leucine supplementation-mediated beneficial effects on aging muscle

3.4.1 mTORC1

Leucine supplementation increases muscle protein synthesis through activating mTORC1 pathway in aging muscle. For example, an earlier study included twenty older participants

showed short-term (4 days) leucine supplementation increased muscle fractional synthesis rate (FSR) (32). However, they did not measure how mTORC1 pathway was regulated by leucine supplementation. A later study demonstrated that chronic leucine supplementation (2 weeks) increased postabsorptive muscle FSR in older adults; at the same time, higher phosphorylation level of mTORC1, 4EBP1, and S6K1 were observed in vastus lateralis muscle from older adults supplemented with leucine (9). Inconsistent results were reported in animal studies. Long-term (6 months) leucine supplementation did not alter mTORC1 pathway in gastrocnemius muscle from 18-month-old rats and muscle mass (54). Another animal study showed leucine-enriched essential amino acid (EAA) mixture supplementation increased phosphorylation level of mTORC1 and muscle fiber size in aged rats (30). In this study, phosphorylation level of 4EBP1 and S6K1 were not measured to verify true activation of mTORC1 pathway. Leucine supplementation also induces muscle hypertrophy and mTORC1 pathway activation in C2C12 myotubes (2). Overall, leucine supplementation can increase muscle protein synthesis through activating mTORC1 pathway in muscle from aging adults; however, this is still unclear in aging animal models and cell models.

3.4.2 Autophagy

Leucine supplementation is shown to reduce muscle protein degradation through suppressing autophagy signal as LC3 lipidation decreased after leucine supplementation in C2C12 myotubes (35). In animal, leucine supplementation was also found to increase muscle mass and inhibit autophagy signal (39). In dexamethasone-induced muscle atrophy animal model, leucine-enriched branched-chain amino acids (BCAA) supplementation reduced LC3 lipidation in muscle (52). While in another study using dexamethasone-induced muscle atrophy animal model, LC3 expression in muscle did not change after leucine supplementation (5). In

addition, unchanged LC3 lipidation was reported in muscle from hindlimb suspended rats that supplemented with leucine-enriched BCAA (24). In the muscle atrophy models, it is still unclear if autophagy is involved in leucine supplementation-mediated muscle improvements. Further, some studies used leucine-enriched BCAA supplementation, instead of leucine supplementation, which did not completely prove the muscle beneficial effects were caused by leucine. More markers for autophagy such as autophagy-related genes (ATGs) expression, autophagosome morphology, lysosomal enzymatic activity, not just LC3 lipidation or LC3 expression. Additionally, there is no study conducted to investigate how autophagy is regulated by leucine supplementation in aging muscle.

3.4.3 Mitochondria

In vitro, it is shown that leucine increased mitochondrial mass, oxygen consumption and expression of mitochondrial biogenesis-related genes including sirtuin1 (SIRT1), PGC-1α, and nuclear respiratory factor 1 (NRF1) in C2C12 myotubes (41). In animal model of aging, muscle mitochondrial COX activity was shown to be enhanced by leucine supplementation, while citrate synthase activity was not changed by leucine supplementation (18). From the *in vitro* and in *in vivo* studies, it is suggested that leucine could improve mitochondrial function and biogenesis in muscle cells and aging muscle. However, the current evidence in clinical trials are inconsistent. Leucine-enriched BCAA supplementation did not change mitochondrial ATP production and mitochondrial DNA copy numbers in vastus lateralis muscle from older adults (42). Similarly, ATP production in muscle from older adults consumed essential amino acid (EAA) supplementation deprived of leucine did not differ from adults consumed EAA supplementation containing leucine (28). Notably, the muscle cells used in previous mentioned *in vitro* study was not considered as “old”. It is shown that high passage C2C12 myotubes had lower mitochondrial

DNA, which suggested lower expression of mitochondria-related proteins (31). Therefore, it is still unknown if leucine can improve mitochondrial function or biogenesis under aging conditions at *in vitro* level. In addition, more markers such as mitochondrial morphology and bioenergetics are needed to be verified for the effect of leucine supplementation on mitochondria in aging muscle.

3.4.4 Fat Metabolism

Leucine increases fatty acid oxidation in skeletal muscles (40). Sun et al showed increased fatty acid oxidation and expression of uncoupling protein 3 (UCP3) in C2C12 myotubes (40). Leucine was also shown to increase the phosphorylation level of acetyl-CoA carboxylase (ACC), an enzyme involved in fatty acid synthesis, which suggested a lower level of fatty acid synthesis (4). Inconsistent results are reported in *in vitro* study (36). For example, increased gene expression of fatty acid synthase (FAS) and cellular lipid content were found in C2C12 myotubes treated by leucine (36). In addition, lipid content measured by oil red o staining was increased by leucine treatment (33). However, another study reported reduced lipid content in C2C12 myotubes treated by both palmitate and leucine (20). *In vitro* study from our lab also showed leucine decreased intracellular lipid droplets in palmitate-treated C2C12 myotubes (51). Notably, these studies all showed increased mitochondria biogenesis and function-related gene expression. As Cpt1 localizes on the outer membrane of mitochondria, mitochondria is also considered to play a role in fatty acid oxidation. Therefore, more studies need to be conduct to understand.

3.5 Conclusion

Impaired pathways involved in compromised muscle performance and reduced muscle mass in the aging population is well-studied. In addition, effect of leucine supplementation on increasing muscle protein synthesis through mTORC1 pathway is also well-recognized. However, the role of protein breakdown-related autophagy pathway, mitochondrial pathway and related fat metabolism are less studied. Therefore, it is necessary to understand whether these pathways are regulated by leucine supplementation in muscle under aging conditions.

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4 Chapter 3. Differential Gene Expression of Autophagy, Mitochondrial Biogenesis and Fat Metabolism in Skeletal Muscle of Overweight and Obese Adults, and Relationship with Whole Body Protein Kinetics

4.1 Abstract

Obesity prevalence keeps increasing in the United States. One of the consequences of obesity on the skeletal muscle is poor physical performance and reduced relative muscle mass. Reduced skeletal muscle physical performance will result in poor life quality of obese population. Therefore, the first objective of this study was to compare the gene expression related to autophagy, the ubiquitin proteasome system, mitochondrial biogenesis, and fat metabolism between normal weight and overweight and obese adults. The second objective was to determine if there was a correlation between whole body protein kinetics and gene expression in individuals of different BMI. RT-qPCR was used to determine the relative fold change of targeted genes in the skeletal muscle of overweight and obese adults. We then performed hierarchical regression analysis to determine if BMI had a moderating effect on the correlation between expression of targeted genes and whole body protein kinetics. We found an increase in expression of autophagy-related genes, ATF4, GADD45A, ATG3, ATG5, ATG7, Beclin1, LC3B, p62, ubiquitin proteasome system-related gene, MuRF1, mitochondrial biogenesis-related genes, NRF1, PGC1α, Tfam, UCP2, and fat metabolism-related genes, ACC, PPARα, SREBP1, FATP1, FATP4, in the overweight and obese adults. In addition, decreases in the expression of ubiquitin proteasome system-related gene, Atrogin1, and mitochondrial biogenesis-related gene, PPARγ, were observed in the overweight and obese adults. Hierarchical regression analysis revealed that there was no significant correlation between expression of target genes and whole body protein kinetics.

4.2 Introduction

According to the Centers for Disease Control, approximate 42.4% of adults in the United States are obese (15). Over-accumulation of fat in obese patient disrupts body composition resulting in a decreased skeletal muscle to body fat ratio (25) resulting in decreased skeletal muscle physical performance (17). For example, obese adults with higher intermuscular lipid content have decreased leg muscle strength, muscle power, and modified physical performance test scores (17). Whole body protein turnover balance affects skeletal muscle mass. It has been demonstrated that obese adults have higher protein breakdown and protein synthesis rates compared to normal weight adults, however overall net balance remained unchanged between normal weight and obese adults (5) (11). There are two pathways largely studied for the regulation of protein breakdown, autophagy and the ubiquitin proteasome system (UPS).

Autophagy is activated when damaged cellular organelles and misfolded proteins are accumulated intracellularly. Increased autophagy flux is reported in the primary skeletal muscle cells taken from obese adults compared with lean adults (2). In addition, autophagy signaling is found upregulated in skeletal muscle of diet-induced obese mice (28). Expression levels of the autophagosome marker, microtubule associated protein 1 light chain 3 beta (LC3B), and central autophagy regulator, Beclin1, are upregulated in the skeletal muscle of diet-induced obese mice (28). Regarding to UPS, proteasome activity is shown to be higher in the primary skeletal muscle cells taken from obese adults compared with lean adults (2). UPS signals in muscle are also shown upregulated in skeletal muscle of obese mice (1). Two muscle-specific UPS E3 ligases, Atrogin1 and MuRF1, were upregulated in the muscle of diet-induced obese mice, and a higher content of ubiquitinated protein was detected in muscle from diet-induced obese mice (1).

However, there is gap in how expression of markers of autophagy and UPS change in skeletal muscle of obese adults.

Energy metabolism in skeletal muscle relies heavily on ATP produced by mitochondria. Mitochondrial dysfunction in skeletal muscle occurs with obesity in adults (20). For example, There is lower activity of complex I, a component of the electron transport chain, and mitochondria size in the skeletal muscle of obese subjects compared to lean adults (20, 33). In recent years, there has been accumulating evidence demonstrating impaired mitochondrial function in skeletal muscle of rodent models of diet-induced obesity (16, 18). For example, decreased expression of (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) PGC1α, a transcription factor regulating mitochondrial biogenesis, was found in skeletal muscle from mice fed with high-fat diet for 8 weeks compared with mice fed with control diet for 8 weeks (18). Further, smaller intermyofibrillar mitochondrial size has also observed in the mice fed with high-fat diet for 4 weeks compared with mice fed with a control diet for 4 weeks (16).

Energy metabolism is also affected by balance between fat oxidation and fat synthesis. Individuals with obesity have increased fat accumulation surrounding and within skeletal muscle (14). This could be due to a decrease in fatty acid oxidation observed in the skeletal muscle from obese adults (24) or a decrease in expression of carnitine palmitoyltransferase 1 (Cpt1), the enzyme that catalyzes the first rate-limiting step of fatty acid oxidation (30). In addition, there is upregulated fat synthesis-related enzyme, stearoyl-CoA desaturase-1, expression in skeletal muscle of obese adults compared with lean adults (19). However, the gene expression related to fat metabolism in skeletal muscle of obese adults are unknown.

Therefore, the first objective of this study was to determine differences in gene expression related to autophagy, UPS, mitochondria, and fat metabolism in the skeletal muscle

between normal weight and overweight/obese adults. Our second objective was to determine whether BMI has a moderating (enhancing or buffering) effect on the correlation between gene expression and whole-body protein kinetics: protein synthesis rate (PS), protein breakdown rate (PB), and net protein balance (NB).

4.3 Materials and Methods

4.3.1 Participants and skeletal muscle biopsies

In the present study, human vastus lateralis muscle biopsies were collected from two previously published studies, and muscle biopsies collected at fasted state were used for this study (21, 22). Briefly, forty two healthy participants including ten normal weight (NW, defined by BMI < 25 kg/m2), thirty one overweight and obese (OW/OB, defined by BMI $\geqslant$ 25 kg/m2) were recruited from the Little Rock area using local newspaper advertisements and flyers posted around the University of Arkansas for Medical Sciences campus and the Little Rock area. Written informed consent was obtained from all participants, and both studies were approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. Exclusion criteria are described in published studies (21, 22). Characteristics of all participants are shown in Table 4.1.

4.3.2 Stable isotope infusion and determination of whole body protein kinetics calculations

The detailed protocol for tracer infusion and whole body protein kinetics are described in previous published studies (21, 22). Briefly, after an overnight fast, a baseline blood sample was taken to determine the background isotope enrichments. Primed continuous infusion of L-2H5-phenylalanine and L-2H2-tyrosine were performed to determine whole body protein synthesis

(PS), protein breakdown (PB), and net balance (NB). A prime dose of L-2H4-tyrosine was injected to reach isotopic equilibrium of L-2H4-tyrosine enrichment derived from L-2H5-phenylalanine infusion. Plasma was processed before determining the enrichments of phenylalanine and tyrosine. The detailed procedure was described in published study (21). Gas chromatography-mass spectrometry (GCMS) was used for the determination of enrichments of phenylalanine and tyrosine. Rate of appearance (Ra) into the plasma of phenylalanine and tyrosine and the fractional Ra of endogenous tyrosine converted from phenylalanine were used for whole body protein kinetics calculations, details and equations were previously described (21, 23).

4.3.3 Real-time quantitative PCR

Muscle RNA were extracted using TRIzol (Invitrogen, Waltham, MA), according to the manufacturer protocol. Next, a Roche Lightcycler 480 system (Roche, Indianapolis, IN) was used to synthesize cDNA based on the RNA concentrations following the manufacturer instructions. A reporter dye that specifically binds to double-stranded DNA, SYBR green (Quanta, Gaithersburg, MD) was used for the relative quantification of gene expression. Forward and reverse primers for all genes were designed by using Primer-BLAST from the National Center for Biotechnology Information (NCBI, Bethesda, MD). All primer sequences are provided in the supplementary table (supplementary table). Genes tested are: nucleoporin 62 (p62), nucleoporin 53 (p53), autophagy related 3 (ATG3), autophagy related 5 (ATG5), autophagy related 7 (ATG7), Unc-51 like autophagy activating kinase 1 (ULK1), Beclin1, Atrogin1, and muscle RING-finger protein 1 (MuRF1), microtubule-associated proteins 1A/1B light chain 3B (LC3B), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), nuclear respiratory factor 1 (NRF1), peroxisome proliferator-activated nuclear

receptor-gamma (PPARγ), transcription factor A mitochondrial (Tfam), uncoupling protein 2 (UCP2), carnitine palmitoyltransferase I (Cpt1), acetyl-CoA carboxylase (ACC), fatty acid transporter 1 (FATP1), fatty acid transporter 4 (FATP4), peroxisome proliferator-activated nuclear receptor-alpha (PPARα), sterol regulatory element binding transcription factor 1 (SREBP1). 18S ribosomal RNA (18S) was used as an internal reference. All primers were ordered from Integrated DNA Technologies (Coralville, IA) and primer sequences are provided in Table 4.6. All samples and controls were analyzed in duplicate. Fold change of target genes of overweight + obese group versus normal weight group were determined using 2-ΔΔCt method (29).

4.3.4 Statistical analysis

Multiple t test was used for comparing the difference between normal weight and OW/OB groups. Holm-Sidak method was used for correcting multiple comparison. Statistical analyzes were performed and figures were plot in Graphpad Prism, version 6 (San Diego, CA, USA). Hierarchical linear regression analysis was performed to test whether AGE moderated the association between mRNA and whole-body protein synthesis, protein breakdown, or net-balance. In step 1, a control variable (Age) was entered to examine its influence on the dependent variables (PS, PB, and NB). In step 2, independent variables (mRNAs) were included to examine the influence of the independent variables on the dependent variable while controlling with the control variable. In step 3, a moderating variable (BMI) was included to examine whether the moderator variable can have a moderating effect on the dependent variable in the relationship between the dependent variable and the independent variable. Lastly in step 4, all two-way interactions (BMI x mRNAs) were introduced to examine whether the moderator variable can have a moderating effect on the dependent variable when it interacts with the independent

variable. In steps 2, 3, and 4, it was considered statistically significant when the delta F p-value was less than 0.05 and delta R2 increased stepwise. Results of step 1, 2, and 3 are shown in the supplementary tables. Statistical analyzes were performed using IBM SPSS statistics, version 23 (Chicago, IL, USA). All significance level was set at 0.05 ($\alpha = 0.05$). All p values that lower than 0.05 were considered as significant.

4.4 Results

4.4.1 Whole body protein kinetics

In Figure 4.1, there was no significant difference in PS, PB, and NB between and within NW and OW/OB group.

4.4.2 Autophagy gene expression and effect of BMI on correlation with whole body protein kinetics

As shown in Figure 3.2, expression of ATG3 ($p < 0.01$), ATG7 ($p < 0.0001$), Beclin1 ($p < 0.0001$), LC3B ($p < 0.0001$), and p62 ($p < 0.0001$) were upregulated in the OW/OB group. There was no correlation between expression of autophagy-related genes and PS or PB, and BMI (Table 4.2). However, negative correlations were found in correlations with NB in expression of ATF4 ($\beta = -0.213$, $p = 0.021$), GADD45A ($\beta = -0.191$, $p = 0.037$), ATG3 ($\beta = -0.207$, $p = 0.023$), ATG5 ($\beta = -0.207$, $p = 0.024$), ATG7($\beta = -0.194$, $p = 0.036$), Beclin1 ($\beta = -0.216$, $p = 0.019$), and p62 ($\beta = -0.205$, $p = 0.024$) (Table 4.2).

4.4.3 Ubiquitin proteasome system gene expression, and effect of BMI on correlation with whole body protein kinetics

Atrogin-1 and MuRF1 expression did not differ between NW and OW/OB groups (Figure 4.3). There was no significant correlation detected between expression of UPS-related genes and PS or PB (Table 4.3). There were negative correlations detected between expression of Atrogin1

(β = -0.202, p = 0.029) and MuRF1 (β = -0.228, p = 0.016) (Table 4.3) and NB. There was no effect of BMI on these correlations.

4.4.4 Mitochondrial biogenesis gene expression, and effect of BMI on correlation with whole body protein kinetics

Expression of NRF1 ($p < 0.0001$) and UCP2 ($p < 0.0001$) were higher in OW/OB group compared to NW (Figure 4.4). There was no correlation between mitochondria biogenesis-related genes and PS or PB (Table 4.4). Expression of NRF1 (β = -0.180, p = 0.049), PGC1α (β = -0.235, p = 0.009), Tfam (β = -0.217, p = 0.016) were negatively correlated with NB (Table 4.4). There was no effect of BMI on these correlations.

4.4.5 Fat metabolism gene expression, and effect of BMI on correlation with whole body protein kinetics

FATP1 ($p < 0.0001$), FATP4 ($p < 0.0001$), ACC ($p < 0.0001$), PPARα ($p < 0.0001$), and SREBP1 ($p < 0.0001$), were upregulated in OW/OB group compared to the NW group (Figure 4.5). Hierarchical regression analysis showed no significant correlation between all gene expressions and PS or PB (Table 4.5). Furthermore, there were negative correlations between expression of PPARα (β = -0.189, p = 0.038), SREBP1 (β = -0.199, p = 0.029) and NB (Table 4.5). There was no effect of BMI on these correlations.

4.5 Discussion

This study is the first to demonstrate that gene expression related to autophagy, mitochondrial biogenesis, fatty acid transport, adipogenesis, and fatty acid synthesis is upregulated in the skeletal muscle from adults with overweight and obesity compared to normal weight adults.

ATF4 was firstly reported to play a critical role in skeletal muscle atrophy during fasting. (8). GADD45A, was upregulated in a skeletal muscle atrophy model induced by denervation, which was independent of ATF4 level (3). Here we show expression of ATF4 and GADD45A does not change in the OW/OB group, compared with NW group (Figure 4.2), suggesting that ATF4-GADD45A axis was not activated in the skeletal muscle of OW/OB group. In addition, it was reported that expression of genes involved in autophagy pathway such as ULK1, ATG5, ATG7, LC3B, p62, Beclin1 were not different in the skeletal muscle between lean and obese adults at fasted state, and there was no difference in the protein contents of ATG7, LC3B-II/I ratio, and p62 (26). Inconsistent to this report, we find genes such as ATG3, ATG7, Beclin1, LC3B, and p62 were upregulated in the muscle from overweight and obese adults (Figure 4.2). Based on the contradictory results between our findings and report from Kruse et al, we suggest that a further investigation of autophagy, such as morphology of autophagosome by localizing the autophagosome membrane marker p62 and LC3B, in the skeletal muscle of obese adults is needed. Overall, we suggested that increased autophagy signal in the overweight and obese adults could be partially responsible for muscle atrophy in obesity reported from other studies.

Two muscle specific E3 ligases, Atrogin1 and MuRF1, are widely reported to be involved in muscle atrophy. Atrophic muscle cells were showed to have higher Atrogin1 expression, while knockdown of Atrogin1 prevented muscle atrophy (27). Another study also demonstrated expression of Atrogin1 and MuRF1 were upregulated in muscle atrophy cell model (37). Under obesogenic condition such as diet-induced obesity, rats fed with high-fat high-sucrose diet for 12 weeks showed lower expression of Atrogin1 in the vastus lateralis muscle, while MuRF1 was not changed (6). Another *in vivo* study showed increased ubiquitin signal and increased expression of Atrogin1 and MuRF1 in the skeletal muscle taken from high-fat diet fed

mice (1). In the present study, we did not find any significant difference in the expression of Atrogin1 and MuRF1 between NW and OW/OB groups (Figure 4.3). We consider the inconsistency between our results and others is due to the difference in the models, as previous studies only examined Atrogin1 and MuRF1 expression in diet-induced obese mice. Based on our findings, we suggest UPS might be the factor contributing to the muscle atrophy in obesity.

Mitochondrial dysfunctions such as decreased complex I activity and mitochondrial area in the skeletal muscle from obese adults were firstly reported by Kelly et al (20). More recently, in an *in vivo* model of diet-induced obesity, obese mice showed higher protein content of COX-IV and cytochrome c than mice fed with normal diet in the skeletal muscle (12). Unchanged UCP2 expression and upregulated UCP3 expression were firstly shown in the skeletal muscle of obese human (35). However, expression of UCP2 was found upregulated in OW/OB group in the present study (Figure 4.5). We suggest the measurement of UCP2 protein content and mitochondrial bioenergetics of obese muscle are needed for further investigation. One study using grape extract as dietary supplementation for obese Zucker rats showed improved mitochondrial respiration of skeletal muscle, accompanied with decreased expression of NRF1 (32). This study suggests that NRF1 plays a negative role in mitochondrial function in skeletal muscle. Our result also showed an increased expression of NRF1 in the OW/OB group. This suggests an elevation in mitochondrial biogenesis. However, mitochondrial content and size measurements are needed to verify this.

It was firstly shown PGC1α coactivated PPARα to promote transcription of mitochondrial fatty acid oxidation-related genes (34). Here, it was shown that PPARα expression was upregulated in the OW/OB adults (Figure 4.5). Activation of fatty acid oxidation in skeletal muscle cells by adiponectin was shown to be mediated by PPARα (36). However, we did not

find any difference in the expression of Cpt1, which is considered as a central regulator for fatty acid oxidation in skeletal muscle, between NW and OW/OB adults. Over-expressing muscle specific Cpt1 in diet-induced obese rats increased fatty acid oxidation in the skeletal muscle (4). Therefore, we suggested unchanged Cpt1 level, which could be shown as lowered Cpt1 enzymatic activity, might cancel the stimulative effect of PPARα upregulation on fatty acid oxidation in overweight and obese adults. Previous *in vivo* study from our lab showed increased expression of fatty acid synthase in the skeletal muscle of obese Zucker rats fed with a low protein diet (9). This indicated a higher signal of fatty acid synthesis in the skeletal muscle under obesogenic conditions. In addition, O'Neill et al reported that increased fatty acid oxidation, induced by AICAR administration, a drug activates AMPK, was not effective in ACC knockin mice (31). Consistent with this, we found elevated expression of ACC in the skeletal muscle of overweight and obese adults. SREBP1 was shown to play an important role in the differentiation of adipocyte (10). Moreover, mice fed with high-fat diet had slight, but not significant, increase in the expression of SREBP1 in skeletal muscle (7). The insignificance could be explained by insufficient sample size as there were only six mice included in the high-fat diet group. In the present study, we found significant upregulation of SREBP1 in the skeletal muscle of overweight and obese adults. Conclusively, we suggested that upregulation of SREBP1 played a role in adipogenesis around skeletal muscle, and upregulated ACC drove higher intramyocellular fatty acid accumulation.

Obese adults had significantly lower whole body and muscle protein synthesis rate versus lean counterparts (13). However, this study did not measure whole body and muscle protein degradation, in which net balance between protein synthesis and breakdown was unknown. Another study measured whole body protein kinetics showed no difference in net balance

between lean and obese adults (11). In present study, we also did not observe any difference in net balance between normal weight and overweight and obese adults. Hierarchical regression analysis revealed that autophagy, UPS, mitochondrial biogenesis, and fat metabolism-related genes were not correlated with whole body protein kinetics (PS, PB, and NB) after introducing BMI as moderator. Therefore, we concluded that these gene expressions could not be used as predictor for whole body protein kinetics.

There are few limitations in present study. First, we only measured the expression of selected genes from two weight groups, differentiated by BMI. The differences observed at transcription level might not be reflected on translational level. Second, we only compared whole body protein kinetics between normal weight and overweight and obese adults, which could not explain muscle protein kinetics.

In conclusion, the present study demonstrated that body weight may impact gene expression related to autophagy, mitochondrial biogenesis, and fat synthesis in skeletal muscle from overweight and obese adults. As the proposed signal scheme shown in Figure 6, we suggested that increased autophagy-related gene expression could contribute to increased muscle protein breakdown. Although higher expression of mitochondrial biogenesis (NRF1 and UCP2) and fatty acid oxidation (PPARα) related genes were observed in overweight and obese adults, unchanged Cpt1 expression could not realize increased fatty acid oxidation (Figure 4.6).

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4.7 Figures

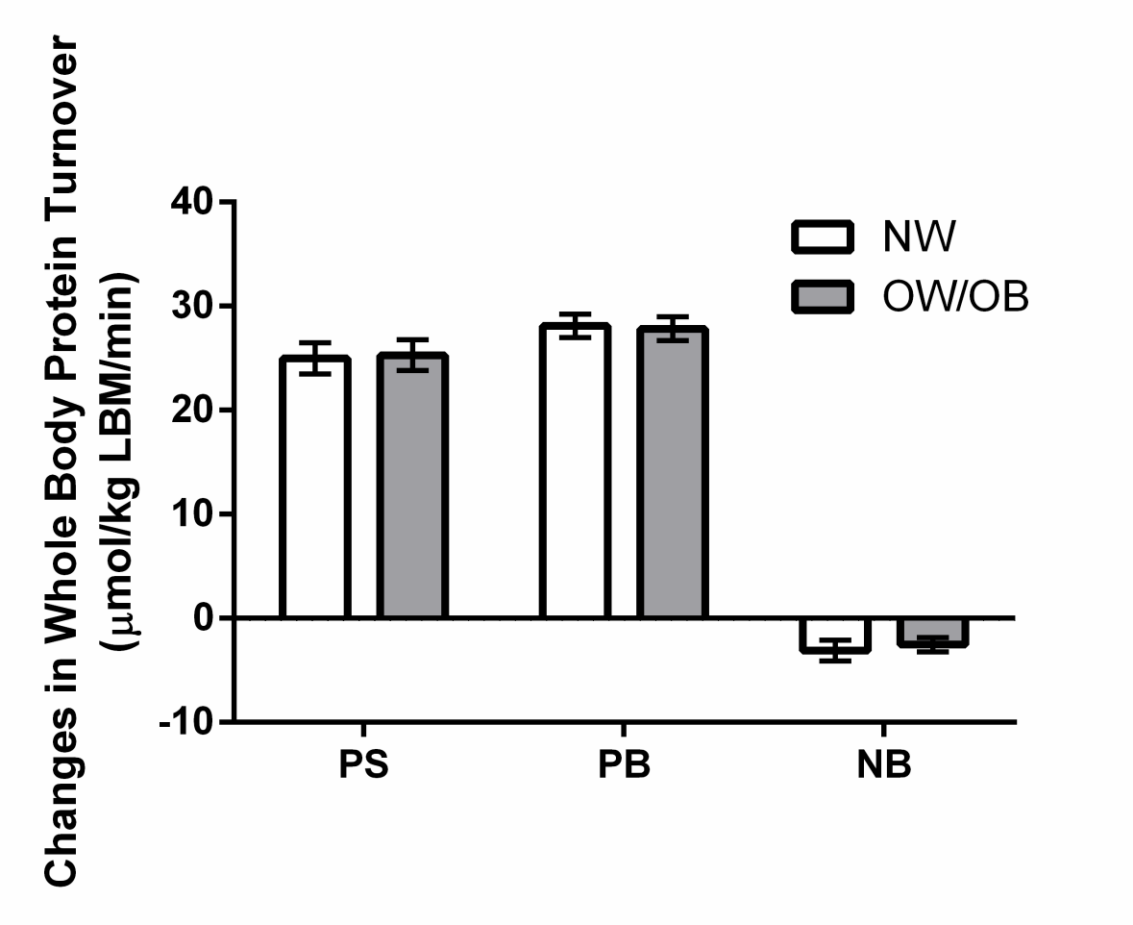

Figure 4.1. Comparison of whole body kinetics between NW and OW/OB groups. PS, protein synthesis; PB, protein breakdown; NB, net balance; NW, normal weight; OW/OB, overweight and obese. *$p < 0.05$. Values are expressed as mean ± SEM. (NW, n = 12; OW/OB: n = 31)

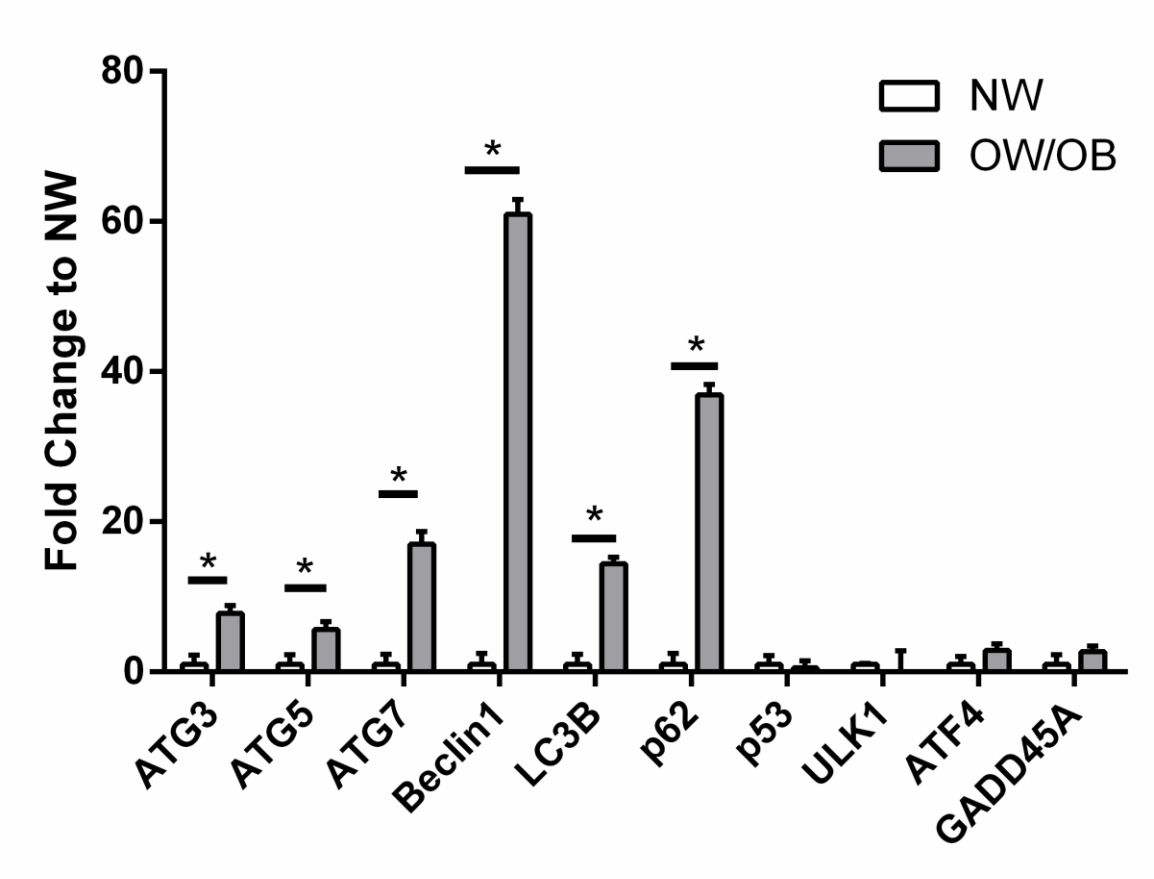

Figure 4.2. Comparison of gene expression of autophagy related genes between NW and OW/OB groups. NW, normal weight; OW & OB, overweight and obese. ATG3, autophagy related 3; ATG5, autophagy related 5; ATG7, autophagy related 7; LC3B, microtubule-associated proteins 1A/1B light chain 3B; ULK1, Unc-51 like autophagy activating kinase 1; p62, nucleoporin 62; p53, nucleoporin 53; ATF4, activating transcription factor 4; GADD45A, growth arrest and DNA damage inducible alpha. *$p < 0.05$. Values are expressed as mean ± SEM. (NW, n = 12; OW/OB, n = 31).

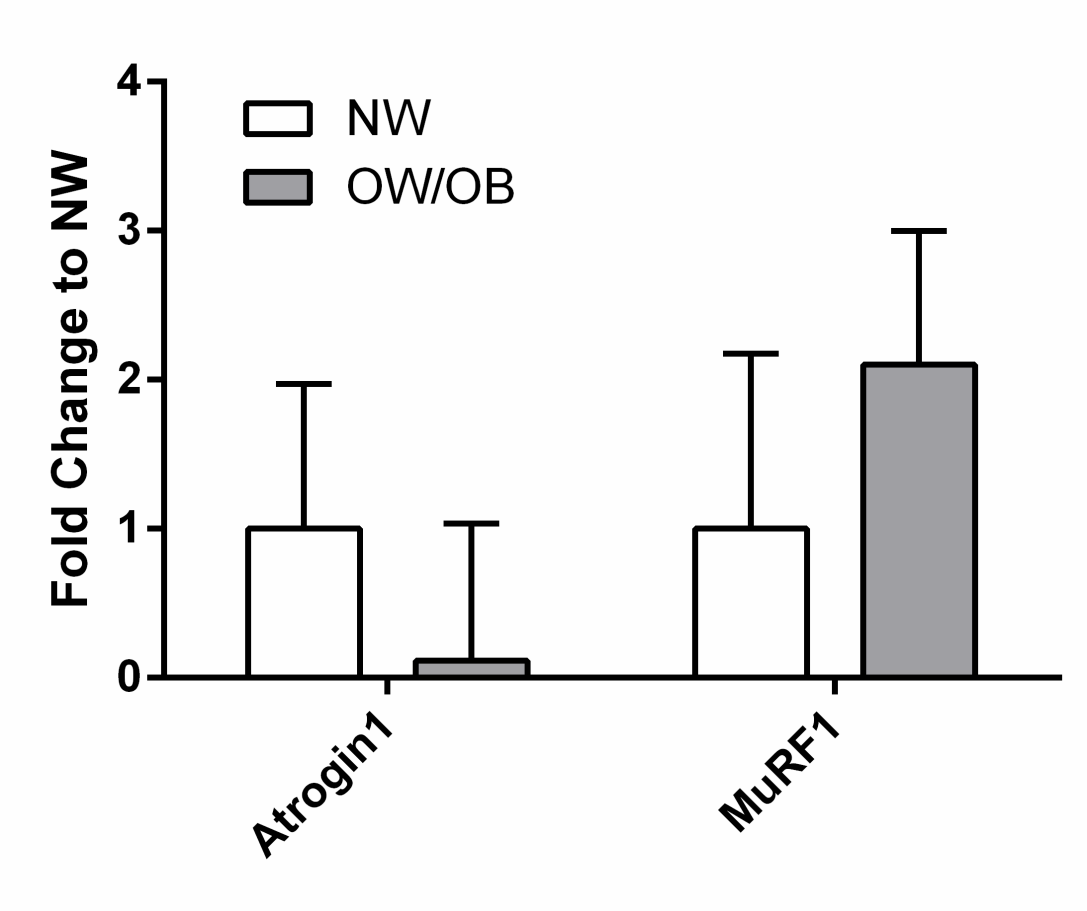

Figure 4.3. Comparison of gene expression of ubiquitin-proteasome system related genes between NW and OW/OB groups. NW, normal weight; OW/OB, overweight and obese. Atrogin1, muscle-specific F-box protein; MuRF1, muscle RING-finger protein-1. *$p < 0.05$. Values are expressed as mean ± SEM. (NW, n = 12; OW/ OB, n = 31)

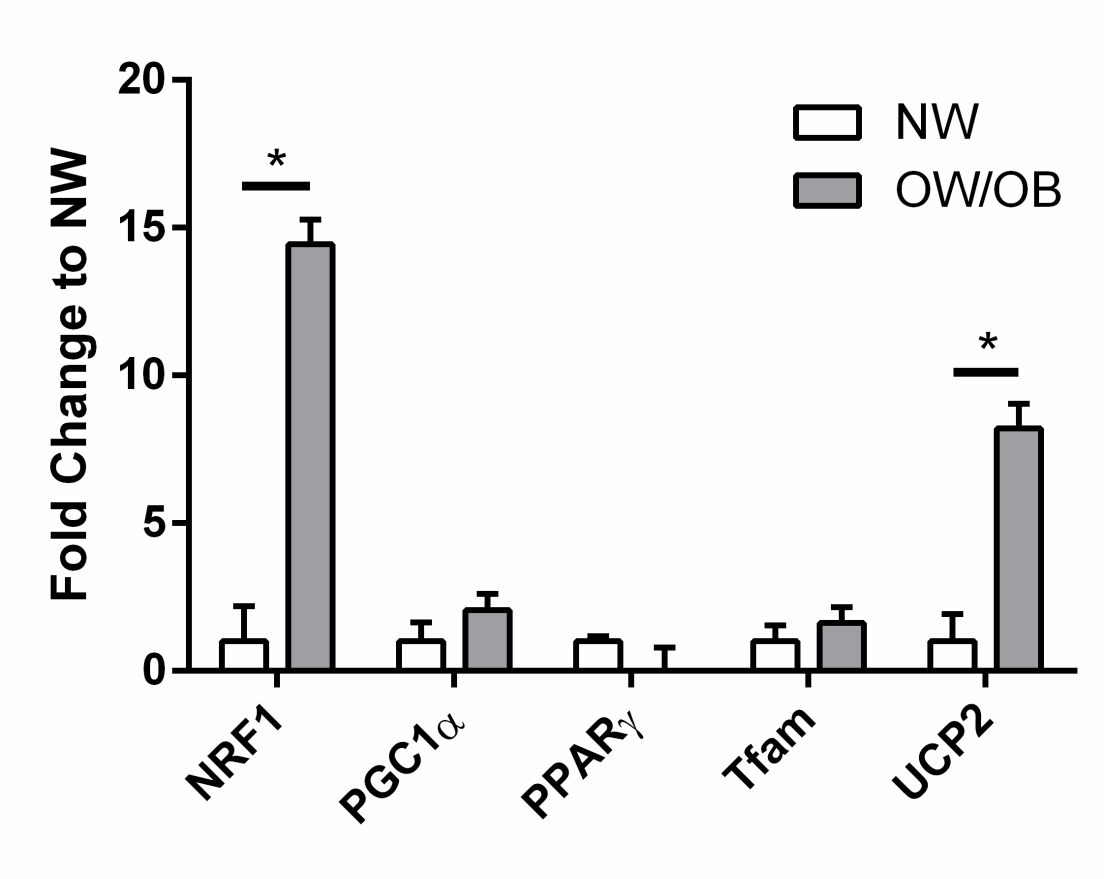

Figure 4.4. Comparison of gene expression of mitochondrial biogenesis related genes between NW and OW/OB groups. NW, normal weight; OW/OB, overweight and obese. NRF1, nuclear respiratory factor 1; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPARγ, peroxisome proliferator-activated receptor gamma; Tfam, mitochondrial transcription factor a; UCP2, uncoupling protein 2. *$p < 0.05$. Values are expressed as mean ± SEM. (NW, n = 12; OW/OB, n = 31)

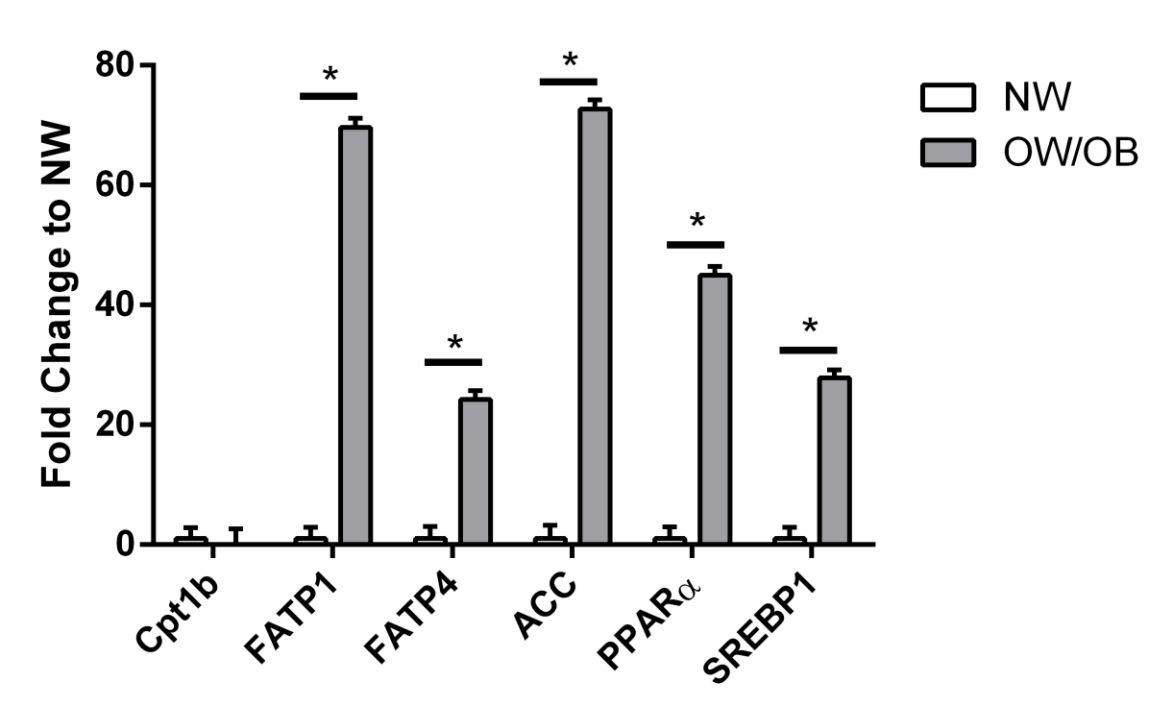

Figure 4.5. Comparison of gene expression of fat metabolism related genes between NW and OW/OB groups. NW, normal weight; OW/OB, overweight and obese. Cpt1b, muscle-specific carnitine palmitoyltransferase 1; FATP1, fatty acid transport protein 1; ; FATP4, fatty acid transport protein 4; ACC, acetyl-CoA carboxylase; PPARα, peroxisome proliferator-activated receptor alpha; SREBP1, sterol regulatory element-binding protein 1. *$p < 0.05$. Values are expressed as mean ± SEM. (NW, n = 12; OW/OB, n = 31)

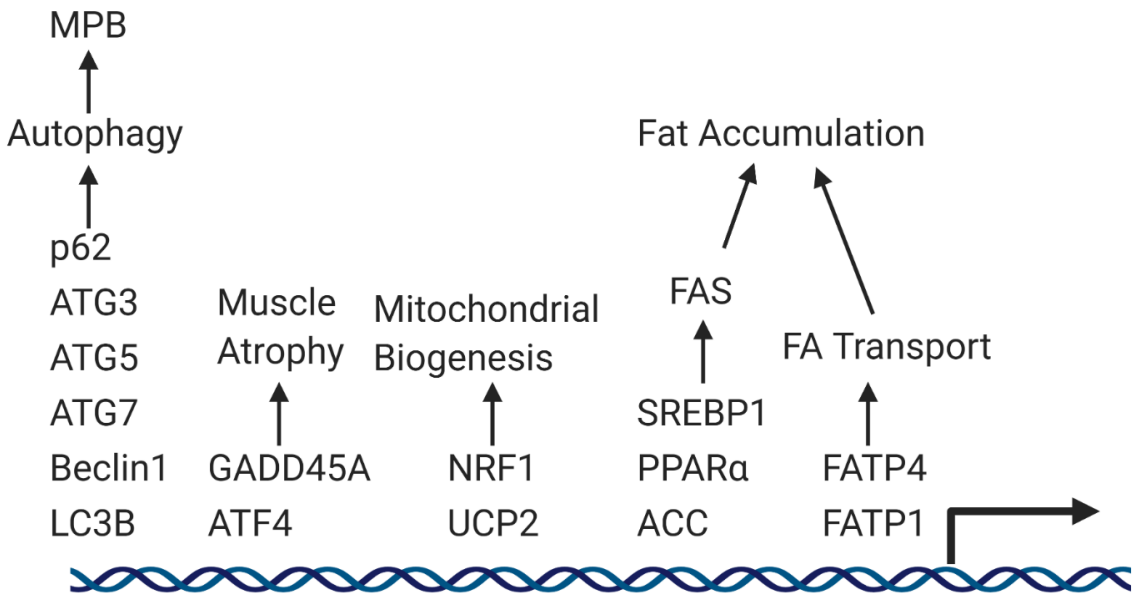

Figure 4.6. Schematic diagram of dysregulated gene expression related to autophagy, UPS, mitochondrial biogenesis, and fat metabolism in the skeletal muscle of obese adults. Upregulated expression of p62, ATG3, ATG5, ATG7, Beclin1, and LC3B in obese muscle might contribute to increase autophagy signaling, and further increases muscle protein breakdown. Elevated GADD45A and ATF4 expression, consistent with their positive role in muscle atrophy, might contribute to muscle atrophy in obesity. Although upregulated NRF1 and UCP2 expression indicated increased mitochondrial biogenesis, no change in muscle-specific Cpt1b explained unchanged fat oxidation. Higher expression of ACC, PPARα, and SREBP1 suggested increased fatty acid synthesis, and higher expression of FATP1 and FATP4 suggested increased fatty acid transport to facilitate fat accumulation in skeletal muscle of obese adults. Figure drawn using Biorender app, https://biorender.com/.

4.8 Tables

Table 4.1. Subjects characteristics.

	NW	OW/OB
Age	43 ± 5.1	47.7 ± 3.4
Sex (F/M)	10/2	11/20
BW, kg	62.9 ± 2.2	89.8 ± 2.3*
BMI, kg/m^2	22.6 ± 0.7	29.2 ± 0.6*
LBM, kg	41.0 ± 1.9	56.3 ± 1.7*
Fat Mass, kg	21.8 ± 1.2	29.0 ± 1.5*

Participants in OW/OB group have higher BW, BMI, LBM, and fat mass than participants in NW group. NW, normal weight; OW/OB, overweight and obese; BW, body weight; BMI, body mass index; LBM, lean body mass. *$p < 0.05$. Values are expressed as mean ± SEM. (NW, n = 12; OW/OB, n = 31)

Table 4.2. Results of hierarchical regression analysis of correlation between autophagy related gene expression and whole body protein kinetics.

PS	ATF4	GADD45A	ATG3	ATG5	ATG7	Beclin1	ULK1	p62	p53	LC3B
Step 1										
β	0.747	0.747	0.747	0.747	0.744	0.755	0.744	0.747	0.747	0.747
R^2	0.558	0.558	0.558	0.558	0.553	0.570	0.554	0.559	0.558	0.558
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 2										
β	-0.142	-0.154	-0.145	-0.161	-0.130	-0.175	0.080	-0.118	-0.076	-0.131
R^2	0.577	0.581	0.579	0.583	0.569	0.600	0.559	0.572	0.564	0.575
p	0.200	0.159	0.183	0.142	0.239	0.109	0.500	0.277	0.487	0.230
Step 3										
β	0.006	0.002	0.222	-0.001	0.002	-0.025	0.025	0.005	0.023	0.000
R^2	0.577	0.581	0.579	0.583	0.570	0.601	0.560	0.572	0.564	0.575
p	0.959	0.982	0.997	0.996	0.985	0.823	0.825	0.965	0.833	0.997
Step 4										
β	0.79	0.785	-0.612	-1.18	-0.977	-1.256	0.327	-0.983	-0.868	-1.332
R^2	0.608	0.604	0.611	0.61	0.59	0.633	0.561	0.595	0.587	0.59
p	0.103	0.156	0.092	0.12	0.193	0.092	0.787	0.189	0.257	0.138
PB										
Step 1										
β	0.396	0.396	0.396	0.396	0.391	0.427	0.412	0.396	0.396	0.396
R^2	0.157	0.157	0.157	0.157	0.153	0.182	0.170	0.157	0.157	0.157
p	0.010*	0.010*	0.010*	0.010*	0.013*	0.007*	0.009*	0.010*	0.010*	0.010*
Step 2										
β	-0.035	-0.066	-0.044	-0.064	-0.034	-0.081	0.079	-0.011	-0.003	-0.031
R^2	0.158	0.161	0.159	0.161	0.154	0.189	0.175	0.157	0.157	0.158
p	0.818	0.663	0.772	0.675	0.827	0.597	0.624	0.942	0.984	0.841
Step 3										
β	-0.078	-0.083	-0.082	-0.084	-0.082	-0.113	-0.076	-0.076	-0.073	-0.080
R^2	0.164	0.168	0.165	0.168	0.161	0.201	0.181	0.162	0.162	0.164
p	0.609	0.586	0.594	0.583	0.600	0.471	0.622	0.620	0.633	0.602
Step 4										
β	-1.19	-1.057	-0.938	-1.107	-0.887	-1.113	0.087	-0.851	-0.645	-1.277
R^2	0.199	0.196	0.185	0.196	0.161	0.229	0.181	0.182	0.173	0.189
p	0.217	0.271	0.35	0.271	0.358	0.27	0.99	0.379	0.505	0.282
NB										
Step 1										
β	0.823	0.823	0.823	0.823	0.820	0.824	0.816	0.823	0.823	0.823
R^2	0.677	0.677	0.677	0.677	0.673	0.679	0.666	0.677	0.677	0.677
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.0001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 2										
β	-0.213	-0.191	-0.207	-0.207	-0.194	-0.216	0.035	-0.205	-0.138	-0.200
R^2	0.720	0.712	0.719	0.718	0.710	0.724	0.667	0.718	0.696	0.716
p	0.021*	0.037*	0.023*	0.024*	0.036*	0.019*	0.732	0.024*	0.137	0.028
Step 3										
β	0.123	0.124	0.177	0.120	0.122	0.115	0.163	0.119	0.147	0.117
R^2	0.735	0.728	0.732	0.732	0.725	0.737	0.693	0.732	0.717	0.729
p	0.157	0.160	0.182	0.171	0.176	0.203	0.093	0.176	0.100	0.188
Step 4										
β	-0.536	-0.425	-0.628	-0.607	-0.54	-0.792	0.499	-0.608	-0.69	-0.644
R^2	0.738	0.729	0.737	0.737	0.728	0.747	0.696	0.734	0.739	0.722
p	0.519	0.624	0.41	0.431	0.49	0.251	0.552	0.411	0.337	0.463

Table 4.3. Results of hierarchical regression analysis of correlation between ubiquitin proteasome system related gene expression and whole body protein kinetics.

PS	Atrogin1	MuRF1
Step 1		
β	0.747	0.744
R^2	0.558	0.553
p	<0.001*	<0.001*
Step 2		
β	-0.159	-0.148
R^2	0.582	0.573
p	0.149	0.191
Step 3		
β	0.027	0.001
R^2	0.583	0.573
p	0.801	0.996
Step 4		
β	-1.274	-1.131
R^2	0.619	0.601
p	0.074	0.128
PB		
Step 1		
β	0.396	0.391
R^2	0.157	0.153
p	0.010*	0.013*
Step 2		
β	-0.066	-0.034
R^2	0.161	0.154
p	0.670	0.831
Step 3		
β	-0.071	-0.081
R^2	0.166	0.161
p	0.639	0.602
Step 4		
β	-1.526	-0.911
R^2	0.228	0.182
p	0.098	0.344
NB		
Step 1		
β	0.823	0.820
R^2	0.677	0.673
p	<0.001*	<0.001*
Step 2		
β	-0.202	-0.228
R^2	0.716	0.721
p	0.029*	0.016*
Step 3		
β	0.153	0.119
R^2	0.739	0.735
p	0.077	0.179
Step 4		
β	-0.69	-0.644
R^2	0.739	0.722
p	0.337	0.463

Table 4.4. Results of hierarchical regression analysis of correlation between mitochondrial biogenesis related gene expression and whole body protein kinetics.

PS	NRF1	PGC1α	PPARγ	Tfam	UCP2
Step 1					
β	0.747	0.747	0.798	0.747	0.747
R^2	0.558	0.558	0.637	0.558	0.558
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 2					
β	-0.110	-0.091	-0.128	-0.126	-0.099
R^2	0.570	0.566	0.652	0.574	0.568
p	0.313	0.408	0.328	0.246	0.366
Step 3					
β	0.009	0.005	-0.009	0.010	0.014
R^2	0.570	0.566	0.652	0.574	0.568
p	0.933	0.966	0.946	0.930	0.897
Step 4					
β	-1.107	-0.903	-0.561	-0.854	-1.136
R^2	0.593	0.585	0.66	0.598	0.592
p	0.165	0.204	0.499	0.149	0.149
PB					
Step 1					
β	0.396	0.396	0.452	0.396	0.396
R^2	0.157	0.157	0.204	0.157	0.157
p	0.010*	0.010*	0.021*	0.010*	0.010*
Step 2					
β	-0.018	0.045	-0.053	-0.012	-0.021
R^2	0.157	0.159	0.207	0.157	0.157
p	0.907	0.767	0.784	0.934	0.889
Step 3					
β	-0.076	-0.066	-0.097	-0.075	-0.075
R^2	0.163	0.163	0.216	0.162	0.163
p	0.619	0.670	0.623	0.624	0.622
Step 4					
β	-0.879	-0.565	-0.374	-0.826	-0.723
R^2	0.179	0.173	0.219	0.192	0.174
p	0.4	0.506	0.756	0.259	0.493
NB					
Step 1 (Age)					
β	0.823	0.823	0.818	0.823	0.823
R^2	0.677	0.677	0.699	0.677	0.677
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 2 (Gene)					
β	-0.180	-0.235	-0.158	-0.217	-0.154
R^2	0.790	0.731	0.692	0.724	0.700
p	0.049*	0.009*	0.201	0.016*	0.096
Step 3 (BMI)					
β	0.127	0.104	0.123	0.126	0.135
R^2	0.725	0.741	0.707	0.739	0.718
p	0.154	0.231	0.310	0.146	0.133
Step 4 (BMI × Gene)					
β	-0.799	-0.87	-0.493	-0.403	-1.077
R^2	0.734	0.754	0.712	0.741	0.738
p	0.271	0.183	0.536	0.617	0.106

Table 4.5. Results of hierarchical regression analysis of correlation between fat metabolisms related gene expression and whole body protein kinetics.

PS	Cpt1	FATP1	FATP4	PPARα	SREBP1	ACC
Step 1 (Age)						
β	0.747	0.747	0.747	0.747	0.747	0.747
R^2	0.558	0.558	0.558	0.558	0.558	0.558
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 2 (Gene)						
β	-0.028	-0.127	-0.138	-0.131	-0.135	-0.126
R^2	0.559	0.574	0.577	0.575	0.576	0.574
p	0.808	0.242	0.206	0.226	0.217	0.245
Step 3 (BMI)						
β	0.022	0.000	0.003	0.002	0.003	0.003
R^2	0.524	0.574	0.577	0.575	0.576	0.574
p	0.840	0.974	0.977	0.984	0.975	0.976
Step 4 (BMI × Gene)						
β	1.201	-1.057	-1.08	-0.98	-1.082	-0.972
R^2	0.531	0.593	0.597	0.593	0.595	0.594
p	0.22	0.2	0.195	0.215	0.181	1.621
PB						
Step 1 (Age)						
β	0.396	0.396	0.396	0.396	0.396	0.396
R^2	0.157	0.157	0.157	0.157	0.157	0.157
p	0.010*	0.010*	0.010*	0.010*	0.010*	0.010*
Step 2 (Gene)						
β	-0.100	-0.045	-0.066	-0.039	-0.036	-0.049
R^2	0.166	0.159	0.161	0.158	0.158	0.159
p	0.522	0.766	0.663	0.798	0.814	0.744
Step 3 (BMI)						
β	-0.077	-0.082	-0.084	-0.081	-0.080	-0.083
R^2	0.172	0.165	0.168	0.165	0.164	0.166
p	0.611	0.595	0.583	0.598	0.603	0.591
Step 4 (BMI × Gene)						
β	1.568	-0.923	-1.013	-0.803	-0.917	-0.77
R^2	0.206	0.182	0.188	0.179	0.182	0.178
p	0.224	0.398	0.351	0.437	0.397	0.445
NB						
Step 1 (Age)						
β	0.823	0.823	0.823	0.823	0.823	0.823
R^2	0.677	0.669	0.677	0.677	0.677	0.677
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 2 (Gene)						
β	0.092	-0.173	-0.162	-0.189	-0.199	-0.164
R^2	0.685	0.706	0.703	0.712	0.716	0.704
p	0.337	0.060	0.079	0.038*	0.029*	0.073
Step 3 (BMI)						
β	0.152	0.124	0.127	0.121	0.121	0.125
R^2	0.708	0.721	0.718	0.727	0.730	0.719
p	0.096	0.166	0.158	0.175	0.169	0.165
Step 4 (BMI × Gene)						
β	-0.019	-0.642	-0.555	-0.67	-0.703	-0.697
R^2	0.708	0.727	0.722	0.733	0.737	0.726
p	0.886	0.408	0.481	0.364	0.331	0.344

Table 4.6. Sequences of primers used for real-time PCR.

Gene Name	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')
Autophagy		
P62	GAGCGGCTCTGGACACCAT	GTGGGCAAAAGTGGTCACAA
P53	TGCAATAGGTGTGCGTCAGAA	CCCCGGGACAAAGCAAA
ATF4	CAGACGGTGAACCCAATTGG	CAACCTGGTCGGGTTTTGTT
GADD45A	GATGTGGCTCTGCAGATCCA	ATGTCGTTCTCGCAGCAAAA
Beclin1	CAAGATCCTGGACCGTGTCA	CCTGGGCTGTGGTAAGTAATGG
LC3B	GGCGCTTACAGCTCAATGCT	TGCTGTGTCCGTTCACCAA
ULK1	AAAGCGAATTTTGTGTGATTTCC	CCCAACAATTCCAAAGGTTTATTT
ATG3	GGGCCGGCCGCTACT	CCAGTGCCTTTCCCTTCACA
ATG5	AAACCCATTCCTTCCAAGCTAGT	GCCAGGGACCACAGTGAAA
ATG7	AGCAGCCCACAGATGGAGTAG	ACGGTCACGGAAGCAAACA
UPS		
Atrogin1	AAGGTAGCGGGTGTGTATTATGC	TCATGGGAAAGGGTATGTGAATC
MuRF1	CAACCTGTGCCGGAAGTGT	CTGGTCCAGTAGGGATTTGCA
Mitochondrial Biogenesis		
PPARγ	GACCACTCCCACTCCTTTGA	GATGCAGGCTCCACTTTGAT
PGC1α	GGAACTGCAGGCCTAACTCC	CACTGTCCCTCAGTTCACCG
NRF1	CCACAGGCAGATGAATGTCTTG	TCCTGGGAAGGAGAGGAGATG
Tfam	ATGCTTATAGGGCGGAGTGG	TGGTTTCCTGTGCCTATCCA
UCP2	TCAGTGCTGGTGGAGTTGACA	GGGATCCTGGCTGGTACGA
Fat Metabolism		
PPARα	GACCACTCCCACTCCTTTGA	GATGCAGGCTCCACTTTGAT
ACC	CATTAGCACAGACATACCT	CACCAATACTCACTTCACT
SREBP1	GTATCAGGCAACTCACTAC	AACATCCATCACTCAACAG
FATP1	TCTTCTGGTCACTACTCA	CCTCGCTCTGTAATCATAA
FATP4	CCTGTTGTTCCTCTACTT	CCTGATGGTCTTGATGAA
Cpt1	TGAGCGACTGGTGGGAGGAG	GAGCCAGACCTTGAAGTAGCG
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

5 Chapter 4. Net Protein Balance is correlated with Increased Expression of Autophagy and Fat Metabolism Genes in Skeletal Muscle from Older Adults

5.1 Abstract

The mechanism of sarcopenia, the main cause for frailty in the older population, is still unclear. Autophagy and the ubiquitin-proteasome system are suggested to play a role in mediating muscle protein breakdown. In addition, compromised muscle performance has been linked with dysfunctional muscular mitochondria. Increased fat deposition and fat infiltration in muscle are also frequently seen in older population. Therefore, the first objective of this study was to compare the gene expression related to autophagy, the ubiquitin proteasome system, mitochondrial biogenesis, and fat metabolism between young and old adults. Our second objective was to determine if there was a correlation between whole body protein turnover and gene expression in individuals of differing ages. We used real-time quantitative PCR to determine the relative fold change of targeted genes in older and young adults. We then performed hierarchical regression analysis to determine if age had a moderating effect on the correlation between expression of targeted genes and whole body protein turnover. Increase in the expression of autophagy related genes, p62, p53, ATG7, and Beclin1, fat metabolism related genes, ACC, FATP1, PPARα, SREBP1, mitochondrial biogenesis related gene, and NRF1 were observed in older adults. A decrease of the mitochondrial biogenesis related gene, Tfam was observed in older adults. In addition, autophagy related genes, ATG3, ATG5, ATG7, Beclin1, p62, p53, ubiquitin proteasome system related gen, MuRF1, mitochondrial biogenesis related genes, NRF1, PGC1α, Tfam, UCP2, fat metabolism related genes, SREBP1, ACC, PPARα, FATP1, were found to be negatively correlated with whole body protein net balance, after age was introduced as moderator. These results suggest that dysregulated gene expression of

autophagy, mitochondrial biogenesis, and fat metabolism could play a role in muscle loss and poor physical performance with aging.

5.2 Introduction

There are approximately 49 million adults aged 65 and older in the United States, and it is estimated that this population will grow to 98 million by 2060 (39). As we age, there is a gradual loss of skeletal muscle mass and function (30), called sarcopenia (23, 49), which may be caused by a decrease in muscle protein synthesis, an increase in muscle protein breakdown, or a combination of those responses (8). For example, myofibrillar protein fractional synthesis rate (FSR) and sarcoplasmic protein FSR are reported to be lower in older adults compared to young adults (15). There is also a negative correlation between age and whole body amino acid kinetics (reflecting protein kinetics) such as phenylalanine and leucine flux with age, which would be consistent with accelerated protein breakdown in aging (46). Impairment of muscle protein synthesis has been shown to be associated with dysregulation of muscle protein synthesis-related signaling in older adults (26). However, no difference in muscle fractional breakdown rate has been observed between young and old adults (22), and this has been further supported by additional studies demonstrating no difference in muscle protein breakdown rates between healthy young and older adults (33, 50). These apparently contradictory findings demonstrate the importance of gaining further understanding into the regulation of muscle protein synthesis and breakdown in older adults.

Activation of the autophagic lysosomal system is related to loss of muscle protein in catabolic states (41). However, the relation of autophagy to muscle loss with aging has not been established (51, 54). Autophagy is usually activated to recycle energy from intracellular damaged organelles and misfolded proteins (37). All misfolded proteins and organelles are engulfed in the

autophagosome, then fused with the lysosome. Degradation of engulfed proteins and organelles recycles usable energy for other pathways, such as protein synthesis, to help cells overcome the crisis state (45). When autophagy occurs in mitochondria, it is called mitophagy (53). Increased occurrence of mitophagy indicates an increase in dysfunctional mitochondria (34). It has been suggested that the accumulation of dysfunctional mitochondria in muscle leads to decreased physical performance (17), and upregulated mitophagy decreases availability of functional mitochondria (13), indicating that higher level of mitophagy or increased autophagy flux might be the potential mechanism in the development of age-related sarcopenia in the skeletal muscle of older adults.

In addition to the possible upregulation of autophagy in sarcopenia, the ubiquitin-proteasome pathway (UPS) may also be upregulated (14). Two major muscle-specific E3 ubiquitin ligases, muscle ring finger 1 (MuRF1) and muscle atrophy F-box (MAFbx, also known as Atrogin-1), are upregulated at the genetic level in human and rats (14, 17) and at protein level in rats under muscle atrophy conditions (2). In addition, two genes were identified to be necessary for mediating muscle atrophy, growth arrest DNA damage-inducible 45a (GADD45A) (6, 12) and activating transcription factor 4 (ATF4) (18, 21). To date, no study compared the expression of Atrogin1, MuRF1, GADD45A, and ATF4 in skeletal muscle of older adults with those of young adults.

Another frequently reported feature of aging skeletal muscle is the imbalance between fat mass and muscle mass within skeletal muscle (27). It has been previously reported that intra-myocellular fat is greater in older adults when compared with their young counterparts (28). It has been suggested that accumulated fat mass, whether infiltrated intracellularly or surrounding the muscle cells, accelerates muscle loss with age (11). Reduced fat oxidation was shown in

muscle of middle-aged adults compared with young adults (9). It is clear that fat oxidation is reduced in skeletal muscle of older adults. However, gene expression related to fat oxidation and fat synthesis are still unknown in the skeletal muscle from older adults.

Therefore, the first objective of this study was to determine differences in gene expression related to autophagy, UPS, mitochondria, and fat metabolism in the skeletal muscle between older and young adults. Our second objective was to determine whether age has a moderating (enhancing or buffering) effect on the correlation between gene expression and whole-body protein kinetics: protein synthesis rate (PS), protein breakdown rate (PB), and net protein balance (NB).

5.3 Materials and Methods

5.3.1 Skeletal muscle biopsies

This study is a secondary analysis of skeletal muscle (vastus lateralis) samples from two previously published studies (35, 36). In total, thirty healthy adults including eleven young (18 – 40 years) and nineteen older adults (52 – 75 years) were recruited from the Little Rock area using local newspaper advertisements and flyers posted around the University of Arkansas for Medical Sciences campus and the Little Rock area. Written informed consent was obtained from all adults, and the study was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. Exclusion criteria is described in the original published article (35). For the current study, we analyzed the baseline muscle samples collected after an overnight fast. Characteristics of all participants can be found in Table 5.1.

5.3.2 Tracer infusion and whole body protein kinetics calculations

The detailed stable isotope tracer infusion protocol and calculations of protein kinetics have been described in previously published papers (35, 36). Briefly, after an overnight fast, two

18-gauge catheters were placed in each lower arm, one for the stable isotope infusion and one for blood sample collection. Primed continuous infusions of L-ring-2H5-phenylalanine (prime, 3.07 μmol/kg; rate, 5.04 μmol·kg-1·h-1) were performed. A priming dose of L-ring-2H4-tyrosine was injected (prime, 0.44 μmol/kg) to reach isotopic equilibrium of L-ring-2H4-tyrosine enrichment derived from L-ring-2H5-phenylalanine infusion. The muscle biopsy used in the present study was taken 2 h after the initiation of the tracer infusion.

Whole body PS and PB rate were calculated based on the determinations of the rate of appearance (Ra) into the plasma of phenylalanine and tyrosine and the fractional Ra of endogenous tyrosine converted from phenylalanine (52). Equations used for calculations were described in published book chapter (52).

5.3.3 Quantitative real-time PCR

RNA samples were isolated using TRIzol (Invitrogen, Waltham, MA), following manufacturer instructions. Then cDNA samples were synthesized based on the concentration of RNA samples in accordance with manufacturer instruction using Roche Lightcycler 480 system. SYBR green master mix (Quanta, Gaithersburg, MD) was used as the reporter dye for mitochondrial biogenesis-related genes: peroxisome proliferator-activated nuclear receptor-gamma (PPARγ), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), transcription factor A mitochondrial (Tfam), uncoupling protein 2 (UCP2), and nuclear respiratory factor 1 (NRF1); fatty acid metabolism-related genes: carnitine palmitoyltransferase I (Cpt1), acetyl-CoA carboxylase (ACC), fatty acid transporter 1 (FATP1), fatty acid transporter 4 (FATP4), peroxisome proliferator-activated nuclear receptor-alpha (PPARα), sterol regulatory element binding transcription factor 1 (SREBP1); autophagy and ubiquitin-proteasome-related genes: nucleoporin 62 (p62), autophagy related 3 (ATG3), autophagy related 5 (ATG5),

autophagy related 7 (ATG7), microtubule-associated proteins 1A/1B light chain 3B (LC3B), nucleoporin 53 (p53), Unc-51 like autophagy activating kinase 1 (ULK1), Beclin1, growth arrest and DNA damage inducible alpha (GADD45A), activating transcription factor 4 (ATF4), Atrogin1, and muscle RING-finger protein 1 (MuRF1); . All primer sequences are provided in Table 5.6. All primers were ordered from Integrated DNA Technologies (Coralville, IA). All samples and controls were analyzed in duplicate. Fold change of target genes of old group versus young group were determined using 2-ΔΔCt method (38).

5.3.4 Statistical analysis

For RT-PCR results, multiple t-test was used for comparing difference between young and old groups. Holm-Sidak method was used for correcting multiple comparisons (1). Statistical analyzes were performed and figures were plot in Graphpad Prism, version 6 (San Diego, CA, USA). Hierarchical linear regression analysis was performed to test whether age affects the association between mRNA and whole-body protein synthesis, protein breakdown, or net-balance. In step 1, a control variable (AGE or BMI) was entered to examine its influence on the dependent variables (PS, PB, and NB). In step 2, independent variables (mRNA) were included to examine the influence of the independent variables on the dependent variable while controlling with the control variable. In step 3, a moderating variable (AGE) was included to examine whether the moderator variable can have a moderating effect on the dependent variable in the relationship between the dependent variable and the independent variable. Lastly in step 4, all two-way interactions (AGE x mRNA) were introduced to examine whether the moderator variable can have a moderating effect on the dependent variable when it interacts with the independent variable. In steps 2, 3, and 4, it was considered statistically significant when the delta F p-value was less than 0.05 and delta R2 increased stepwise. All results of step 1, 2, 3

were provided in supplementary data. Statistical analyzes were performed using IBM SPSS statistics, version 23 (Chicago, IL, USA). All significance level was set at 0.05 ($\alpha = 0.05$). All p values that lower than 0.05 were considered as significant.

5.4 Results

5.4.1 Whole body protein kinetics

As shown in Figure 5.1, older adults had lower PS than young adults ($p < 0.0001$). Older adults showed a decrease in both PS and PB compared with young adults ($p < 0.01$). The NB of older adults was significantly lower than young adults ($p < 0.01$).

5.4.2 Autophagy gene expression, and effect of age on correlation with whole body protein kinetics

Expression of autophagy genes in young versus old muscle are shown in Figure 5.2. There was an upregulation in the gene expression of p62 ($p < 0.001$), ATG7 ($p < 0.0001$), Beclin1 ($p < 0.01$), and p53 ($p < 0.0001$) in the older adults, compared with young adults. There was no significant moderating effect of age on gene expression in relation to PS or PB (Table 5.2). However, the model indicates that age had an effect on the negative correlation between NB and the expression of ATG3 ($p < 0.05$), ATG5 ($p < 0.05$), Beclin1 ($p < 0.01$), and p62 ($p < 0.01$) (Table 5.2).

5.4.3 Ubiquitin-proteasome system gene expression, and effect of age on correlation with whole body protein kinetics

MuRF1 and Atrogin-1 showed no significant difference between older and young adults (Figure 5.3). The hierarchical regression analysis indicates that age had an enhancing effect on the negative correlation between MuRF1 and NB (Table 5.3) ($p < 0.05$).

5.4.4 Differential mitochondrial gene expression, and effect of age on correlation with whole body protein kinetics

NRF1 was higher in the older adults ($p < 0.01$), whereas Tfam was lower in the older adults ($p < 0.05$), compared with young adults (Figure 5.4). NRF1 ($p < 0.01$), PGC-1α ($p < 0.05$), Tfam ($p < 0.05$), and UCP2 ($p < 0.05$) were negatively correlated with NB, and age had an enhancing effect on these correlations (Table 5.4).

5.4.5 Fatty acid metabolism gene expression in young and older participants, and effect of age on correlation with whole body protein kinetics

FATP1, an insulin-sensitive fatty acid transporter, was upregulated in the older adults compared with young adults ($p < 0.001$) (Figure 5.5). Age had an enhancing effect on the correlation between FATP1 and NB ($p < 0.05$) (Table 5.5). The expression of the adipogenesis related gene, PPARα ($p < 0.0001$) was significantly higher in the older adults compared with young adults (Figure 5.5). However, age had no moderating effect on the relationship between PPARα and PS or PB (Table 5.5). For NB, there was a negative correlation between PPARα and NB. Moreover, there was a moderating effect on the relationship from age ($p < 0.05$) (Table 5.5). The fatty acid biogenesis-related genes, SREBP1 ($p < 0.01$) and ACC ($p < 0.01$) were upregulated in the older adults (Figure 5.5). Hierarchical regression analysis showed that ACC was negatively correlated with PS, while age had an enhancing effect on the correlation between

ACC and PS ($p < 0.05$) (Table 5.5). Both SREBP1 ($p < 0.05$) and ACC ($p < 0.05$) were negatively correlated with NB. In addition, age had an enhancing effect on both correlations (Table 5.5).

5.5 Discussion

To our knowledge, this is the first studies to demonstrate that expression of genes related to autophagy, UPS, mitochondrial biogenesis, and fat metabolism are differentially regulated in the skeletal muscle of young and older adults. In addition, this is the first study to our knowledge to examine the correlation between age, whole-body protein kinetics, and gene expression related to protein synthesis, protein breakdown, and energy metabolism. While the body of research examining skeletal muscle gene expression in young versus older rodents is abundant, the available data from young and older human skeletal muscle is lacking. This study helps fill this research gap.

In the present study, markers of autophagy such as p62, ATG7, Beclin1, and p53, were upregulated in the skeletal muscle of older adults compared with young adults. This is supported by studies in skeletal muscle taken from older adults showing upregulated expression of p62 (42), and aging mice had higher cytosolic expression of p62 and Beclin1 (44). Consistent with the mice study (44), we found no differences in LC3B expression in older adults compared with young adults. One group of researchers showed that overexpression of GADD45A in the tibialis anterior muscle of healthy mice developed significant muscle atrophy (12). However, we found no difference in expression of GADD45A and ATF4 between young and older adults. In addition, depletion of ATF4, an upstream of GADD45A, reduced the level of muscle atrophy induced by fasting in mice (19). This discrepancy could be explained by the difference between

artificial skeletal muscle atrophy in model animal and age-related skeletal muscle dysfunction in humans. Interpreting our data in the context of the results reported by Sakuma et al (44), we suggest that autophagy could be dysregulated, especially upregulation of p62 and Beclin1. Over-expression of these genes would facilitate the formation of autophagosome in the skeletal muscle in aged individuals, which could contribute to muscle loss. At the same time, the gene expression patterns of GADD45A and ATF4 under aging scenario need more investigation. Notably, there is a discrepancy exists between upregulated autophagy gene expression and decreased PB in older adults, compared with young adults. There are other multiple potential reasons for decreased PB in older adults: 1) physiologically, autophagy flux is affected by formation of autophagosome, infusion of autophagosome and lysosome, and the activity of lysosomal enzymes; 2) besides autophagy and UPS contribute to protein breakdown, calpain Ca2+-dependent cysteine proteases also regulates protein breakdown; 3) the PB we are demonstrating here is whole body protein breakdown rate, however, the pattern of muscle protein breakdown rate could be different with whole body protein breakdown rate.

E3 ubiquitin ligase is universally accepted as the central regulator of the UPS process (4). Overexpressed E3 ubiquitin ligase would result in over-activation of UPS and unnecessary protein degradation (40). Atrogin1 (24) and MuRF1 (10) are two well-established skeletal muscle specific E3 ubiquitin ligases, which have been shown to be highly upregulated in mice with muscle atrophy (47). Interestingly, there are contrasting results reported on Atrogin1 and MuRF1 expression under muscle atrophy conditions such as suppressed Atrogin-1 expression in soleus, medial gastrocnemius, and tibialis anterior muscles of hindlimb unloaded mice (7). In addition, Atrogin1 and MuRF1 expressions were upregulated in sedentary men underwent forty-eight hours of unloading by unilateral lower limb suspension, a procedure to induce muscle

disuse-related atrophy (43). Another *in vivo* model of age-related muscle loss showed downregulated expression of Atrogin1 and MuRF1 in the gastrocnemius muscle (20). In this study, we did not find differences in expressions of either Atrogin-1 or MuRF1 between young and older adults. Differences in inducing skeletal muscle loss in the animal models (such as dietary restriction-induced atrophy vs. age-related atrophy) could explain the contradictory results in the expression patterns of Atrogin1 and MuRF1. Refer to our result, although no difference in neither Atrogin1 nor MuRF1 expression was found, the enzymatic activity would be different between two age groups.

Mitochondrial dysfunction has been widely reported in the skeletal muscle of older population. It was shown that pre-frail older adults had lower rate of ATP production, lower abundance of mitochondrial respiratory complex I, IV, and V and lower enzymatic activity of complex I, II and IV than healthy active older adults (3). Similarly, reduced cytochrome c oxidase (COX) activity and decreased expression of muscle specific PGC1α and COX I were found in the low-functioning older adults (32). In the present study, we found that expression of PGC1α and PPARγ, two important transcription factors mediating mitochondrial biogenesis, did not differ between young and older adults. NRF1 deficiency was shown to exacerbate mitochondrial biogenesis impairment in aging animals (29). Inconsistent with this result, a higher NRF1 expression was observed in the older adults in the present study. These findings suggest that possible impairments in translational machinery in the skeletal muscle of older adults might not be able to translate upregulated NRF1 mRNA into functional protein. The other finding in the Figure 3, decreased Tfam expression would account for the discrepancy of increased NRF1 expression in older adults, since muscle fiber, under age-related muscle loss condition, might prefer Tfam to regulate mitochondrial biogenesis, not NRF1. In addition, more investigation on

NRF2, which often functions together with NRF1 to facilitate key mitochondrial metabolic gene regulation, is needed.

Increased fat deposition in aged skeletal muscle has been frequently reported (48). It was previously shown that ACC, the rate-limiting enzyme for fatty acid synthesis, was upregulated in the skeletal muscle of older adults, which was in line with our finding. In addition, PPARα is well known for mediating the activation of adipocyte differentiation for adipogenesis (25). Here, we also found upregulated level of PPARα gene in the skeletal muscle from older adults. Therefore, we suggest that elevated fat deposition could be the results of enhanced fatty acid synthesis and adipogenesis activation caused by ACC and PPARα upregulation. FATP1 and FATP4, two skeletal muscle abundant fatty acid transport proteins, were reported to be differentially expressed in the muscle of exercised healthy young adults (31). In another *in vivo* model of muscle hypertrophy, myostatin knocked out mice, density of muscle FATP1 and FATP4 proteins were lower than wild type (5). These studies suggest FATP1 and FATP4 are upregulated with muscle growth. However, we found completely opposite results that FATP1 and FATP4 upregulated in the skeletal muscle of older adults. But notably, despite the fact that upregulated FATP1 and FATP4 level would suggest higher fatty acid transport capacity for oxidation, unchanged gene expression of Cpt1, the rate-limiting enzyme for fatty acid oxidation, between young and old adults in our study would explain the deficiency in fatty acid catabolism reported elsewhere. As a transcription factor that regulates fatty acid synthesis in lipogenic tissues, overexpression of SREBP1 was also shown to induce muscle atrophy and decreases protein synthesis rate in an *in vitro* model of human skeletal muscle (16). Consistent with this, we found upregulated SREBP1 level in the skeletal muscle of older adults. Therefore, we suggest that increased fatty acid synthesis-related gene expression might be responsible for the over-

accumulation of fat observed in the skeletal muscle, not fatty acid oxidation, although expression of fatty acid transport genes was elevated.

It is well established that older adults have a lower ratio of whole body protein synthesis rate to whole body protein breakdown rate, i.e., negative net protein balance (46). We also verified this in the present study. Although we did not find any correlation between autophagy gene expression and PS or PB, or any moderating (enhancing or buffering) effect of age on this correlation, we did observe negative correlations between NB and gene expressions such as ATG3, ATG5, ATG7, Beclin1, p53, and p62 when age was introduced as moderating variable. This indicates that when age interacted with these gene expressions, the negative correlations were enhanced from non-significant to significant. In addition, after age was introduced, the negative correlation between MuRF1 expression and NB became significant. This indicates that age had an enhancing effect on the association between MuRF1 and NB. Furthermore, the negative correlation between NB and mitochondrial biogenesis-related genes such as NRF1, PGC1α, Tfam, and UCP2 became significant after age was introduced, which suggested that age, as a moderator, enhanced the negative correlations. Finally, there were negative correlations between NB and fatty acid metabolism-related genes such as FATP1 (fatty acid oxidation), PPARα (adipogenesis), and ACC, SREBP1 (fatty acid synthesis) after age was introduced. As a conclusion, we suggest that whole body protein kinetics in older adults tend to shift to higher protein breakdown and lower protein synthesis, and this should account for the muscle loss. Multiple mechanisms should be responsible for this shift. Our data showed that older adults had higher autophagy, adipogenesis and fatty acid synthesis gene expression than young adults. Besides, autophagy, UPS, mitochondrial biogenesis, fatty acid oxidation, adipogenesis, and fatty

acid synthesis gene expression could function as good negative predictor for whole body protein net balance.

There are several limitations to this study. First, we only measured the gene expression of selected genes of two age groups. Any difference found on the transcription level might not be reflected on the protein level or protein functional level. Second, we only compared the differences in whole body protein kinetics between young and older adults. Volpi et al reported the muscle protein fractional synthesis rate was not different between young and old adults (50). However, they did not measure muscle protein breakdown rate, which could be a technical challenge in measuring protein breakdown rate over a decade ago. Third, we only determined the correlations between whole body protein kinetics and expression of genes related to selected pathways, and the correlations did not completely reflect the causality.

In conclusion, the proposed schematic diagram is present in Figure 6. In addition, this is the first study to examine the correlation between gene expression related to protein breakdown, mitochondrial biogenesis, fat metabolism and whole body protein kinetics.

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5.7 Figures

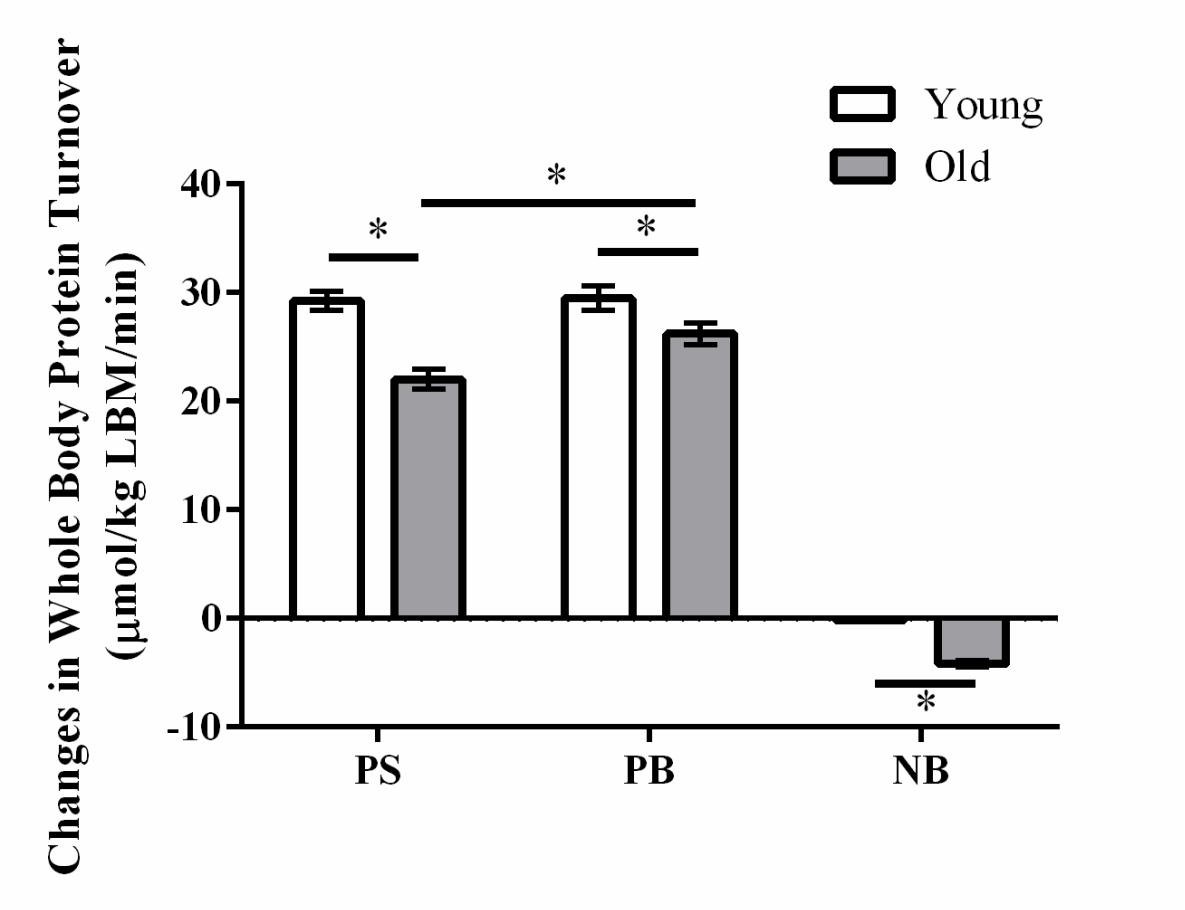

Figure 5.1. Whole body protein kinetics in skeletal muscle from young and old participants. Older participants had lower PS and PB than young participants. Within older participants, PS was lower than PB. Older participants had lower NB than younger participants. PS, protein synthesis rate; PB, protein breakdown rate; NB, net balance; LBM, lean body mass. Values represent mean ± SE (young: n = 11, old: n = 19; *, $p < 0.05$; Student t test with Holm-Sidak method).

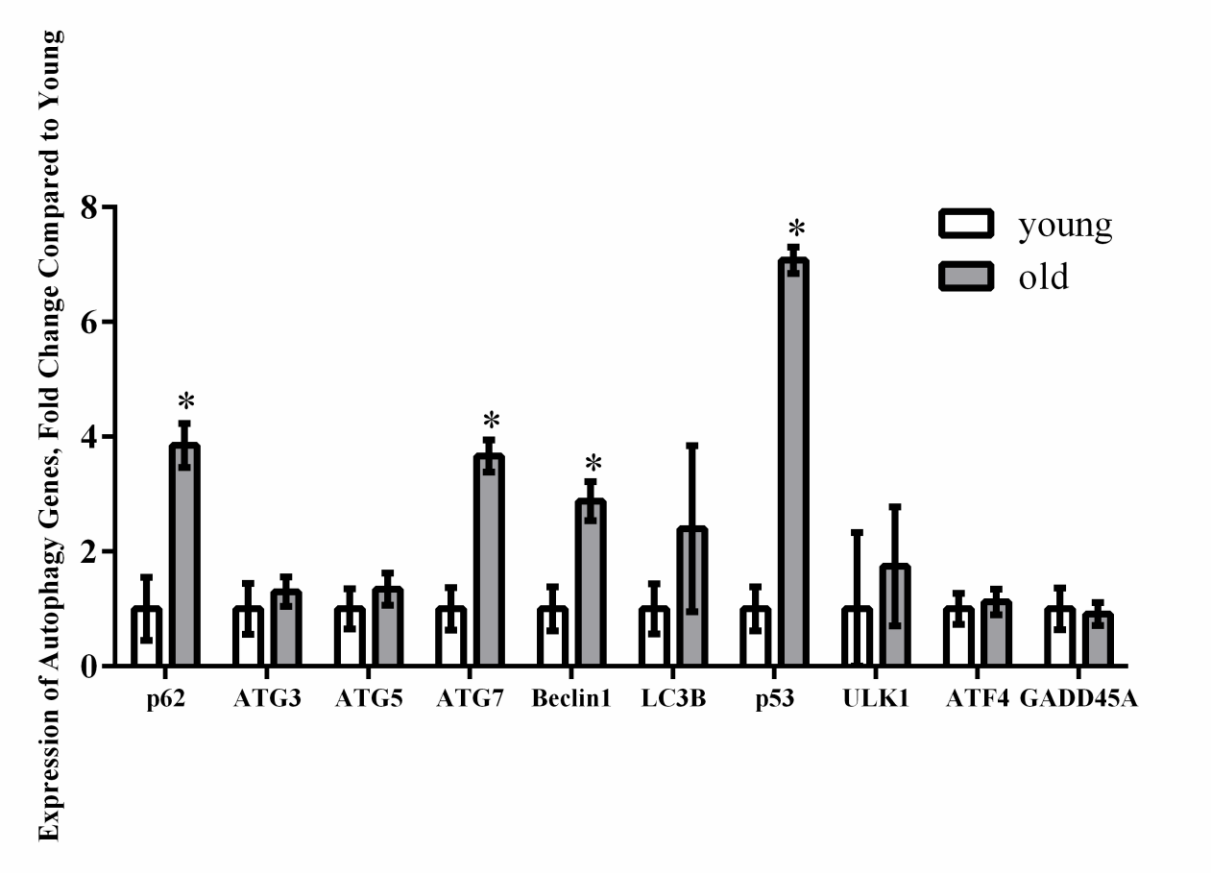

Figure 5.2. Expression of autophagy genes in skeletal muscle from young and old participants. Vastus lateralis muscle biopsies were taken from young and old participants after an overnight fasting. Expression of p62, ATG7, Beclin1, and p53 were found higher in the skeletal muscle of older participants, compared with younger participants. P62, nucleoporin 62; ATG3, autophagy related 3; ATG5, autophagy related 5; ATG7, autophagy related 7; LC3B, microtubule-associated proteins 1A/1B light chain 3B; p53, nucleoporin 53; ULK1, Unc-51 like autophagy activating kinase 1; GADD45A, growth arrest and DNA damage inducible alpha; ATF4, activating transcription factor 4. Values represent mean ± SE (young: n = 11, old: n = 19; *, $p < 0.05$; Student t test with Holm-Sidak method).

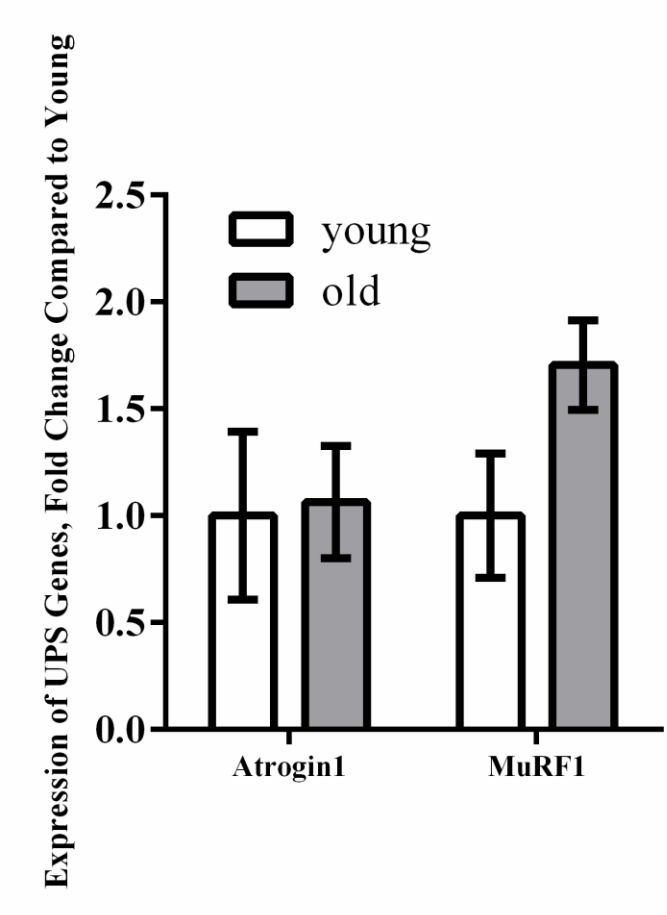

Figure 5.3. Expression of UPS genes in skeletal muscle from young and old participants. Vastus lateralis muscle biopsies were taken from young and old participants after an overnight fasting. No difference was found in expression of Atrogin1 and MuRF1 in skeletal muscle of young and old adults. Atrogin1, muscle atrophy F-box; MuRF1, muscle RING-finger protein 1. Values represent mean ± SE (young: n = 11, old: n = 19; Student t test with Holm-Sidak method).

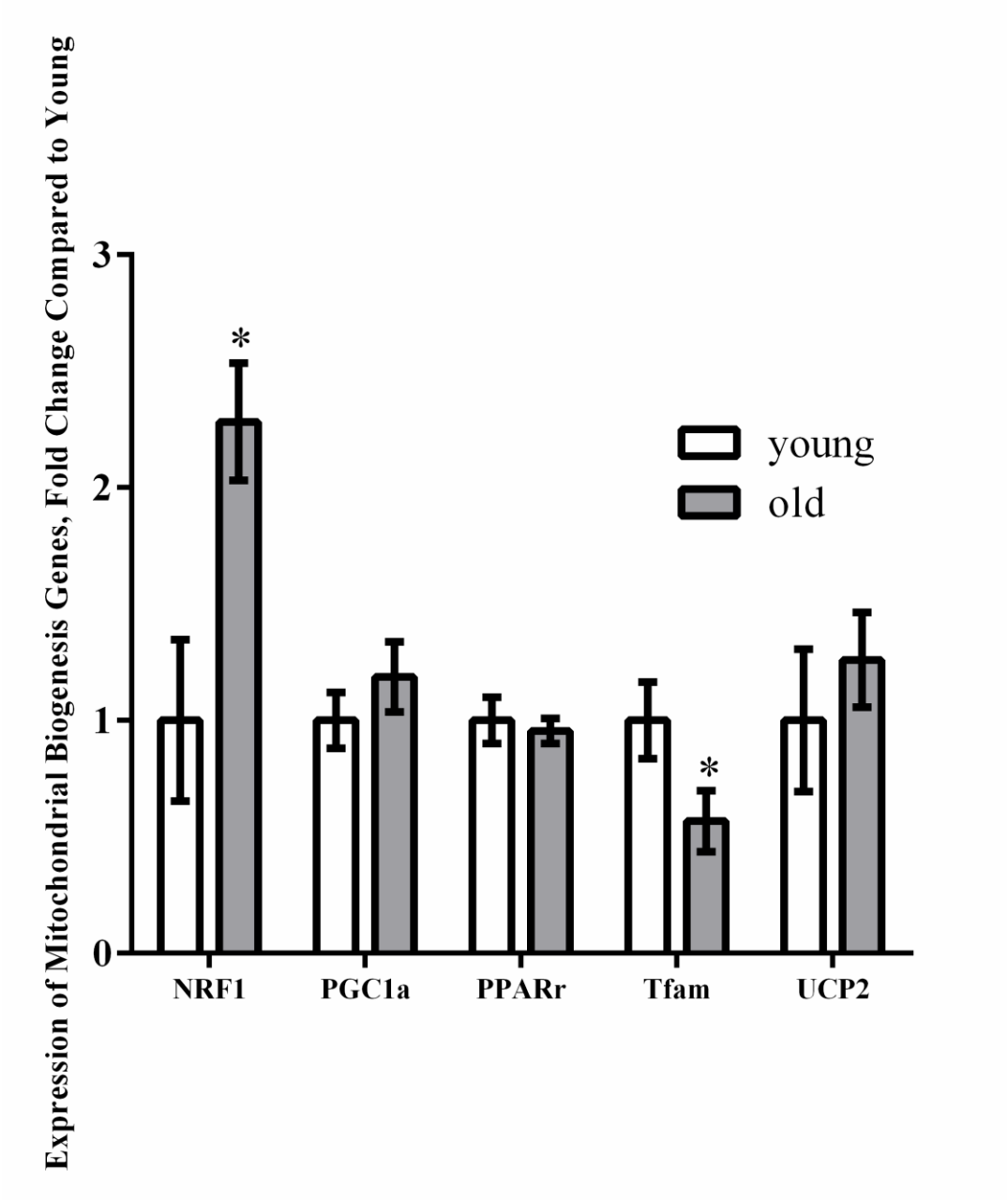

Figure 5.4. Expression of mitochondrial biogenesis genes in skeletal muscle from young and old adults. Vastus lateralis muscle biopsies were taken from young and old participants after an overnight fasting. An increase in the expression of NRF1 and a decrease in the expression of Tfam were found in the skeletal muscle of old adults. PPARγ, peroxisome proliferator-activated nuclear receptor-gamma; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Tfam, transcription factor A mitochondrial; UCP2, uncoupling protein 2; NRF1, nuclear respiratory factor 1. Values represent mean ± SE (young: n = 11, old: n = 19; *, $p < 0.05$; Student t test with Holm-Sidak method).

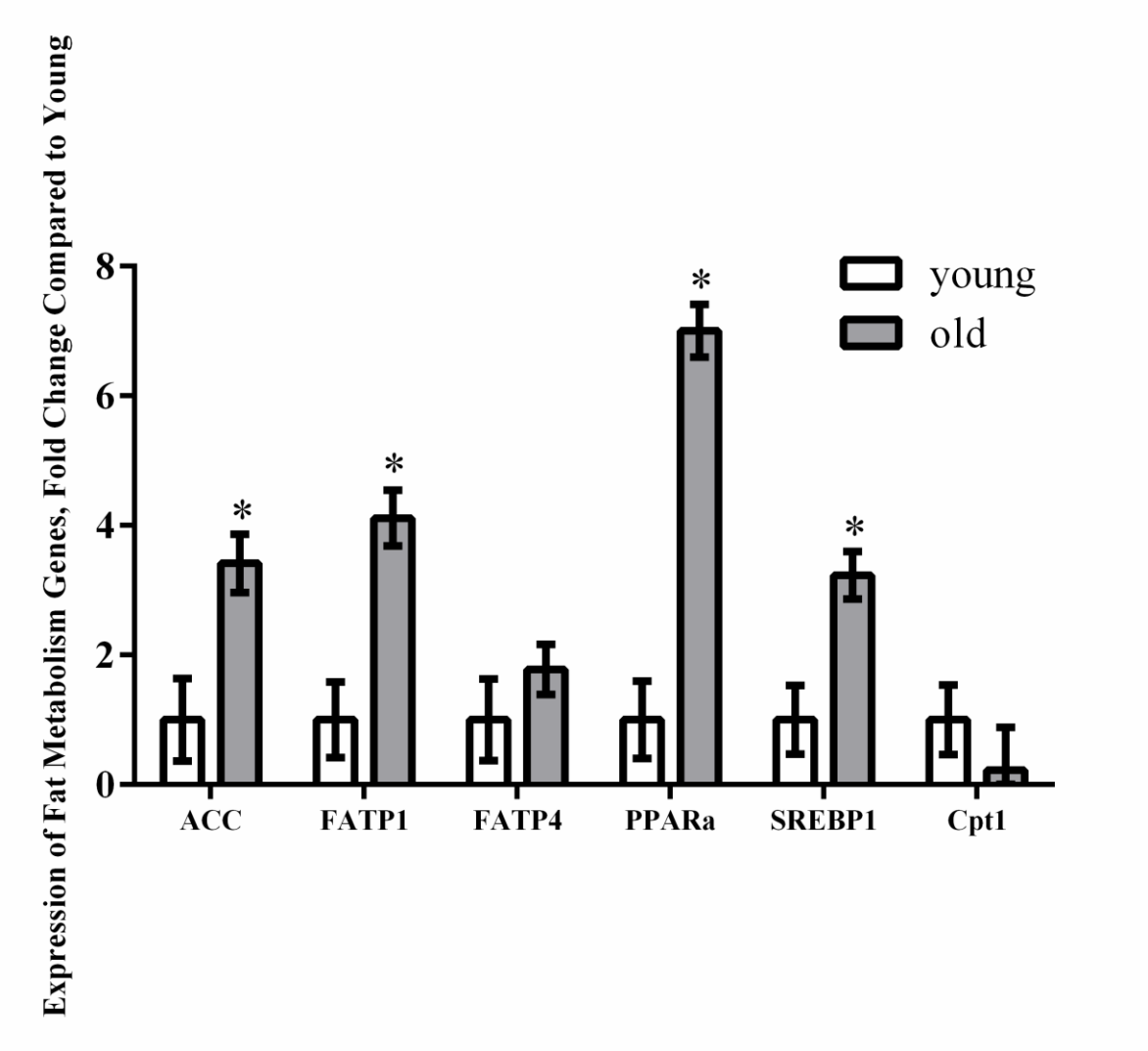

Figure 5.5. Expression of fat metabolism genes in skeletal muscle from young and old participants. Vastus lateralis muscle biopsies were taken from young and old participants after an overnight fasting. Upregulation in expression of ACC, FATP1, PPARα, and SREBP1 were found in the skeletal muscle of old adults. Cpt1, carnitine palmitoyltransferase I; ACC, acetyl-CoA carboxylase; FATP1, fatty acid transporter 1; FATP4, fatty acid transporter 4; PPARα, peroxisome proliferator-activated nuclear receptor-alpha; SREBP1, sterol regulatory element binding transcription factor 1. Values represent mean ± SE (young: n = 11, old: n = 19; *, $p < 0.05$; Student t test with Holm-Sidak method).

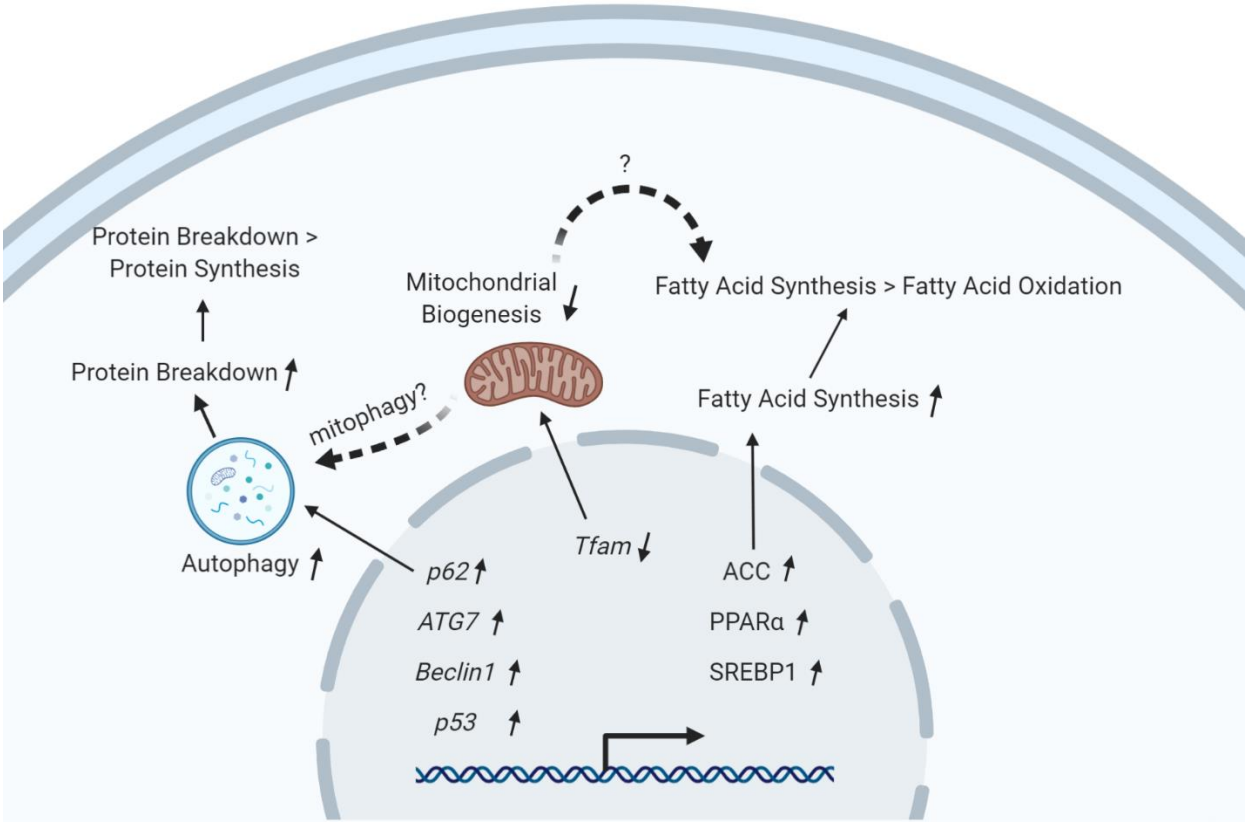

Figure 5.6. Schematic diagram showing dysregulated pathways in the skeletal muscle from older participants Upregulated expression of p62, ATG7, Beclin1, and p53 might contribute to increased autophagy to further increased protein breakdown and mitophagy. Therefore, net balance decreased in the skeletal muscle of older adults. Decreased number of mitochondria might cause by decreased expression of Tfam and increased mitophagy in older adults. Increased fat deposition in the skeletal muscle of older adults could be explained by upregulation of fatty acid synthesis-related genes such as ACC, PPARα, and SREBP1, while expression of fatty acid oxidation-related gene, Cpt1, remained unchanged. Figure drawn using Biorender app, https://biorender.com/.

5.8 Tables

Table 5.1. Participant characteristics.

	Young	Old
Age	31.0 ±1.5	65.5 ± 1.4
Female/Male	7/4	9/10
Body Weight, kg	82.7±1.6	81 ± 3.4
BMI, kg/m^2	26.0 ± 3.5	28.0 ± 0.7
LBM, %	67.1 ± 1.6	61.4 ± 1.8*
Fat Mass, %	27.7 ± 1.7	34.6 ± 1.5*

Values are expressed as mean ± SE. BMI, body mass index; LBM, lean body mass. Older subjects had lower percent LBM and higher fat mass than young subjects (*, $p < 0.05$).

Table 5.2. Hierarchical regression analysis of correlation between autophagy gene expression and whole body protein kinetics.

PS	ATF4	GADD45A	ATG3	ATG5	ATG7	Beclin1	ULK1	p62	p53	LC3B
Step 1										
β	0.048	0.048	0.048	0.048	0.038	0.034	0.046	0.048	0.048	0.048
R^2	0.002	0.002	0.002	0.002	0.001	0.001	0.002	0.002	0.002	0.002
p	0.764	0.764	0.764	0.764	0.816	0.836	0.719	0.764	0.764	0.764
Step 2										
β	0.047	0.003	0.007	0.010	0.013	0.060	0.317	0.004	0.049	0.008
R^2	0.004	0.002	0.002	0.002	0.002	0.005	0.102	0.002	0.005	0.002
p	0.775	0.987	0.968	0.951	0.940	0.724	0.053	0.981	0.765	0.964
Step 3										
β	0.780	0.777	0.774	0.781	0.763	0.781	0.717	0.765	0.759	0.769
R^2	0.577	0.581	0.579	0.783	0.570	0.601	0.560	0.572	0.564	0.575
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 4										
β	-0.558	-0.612	-0.652	-0.639	0.292	-0.592	-0.682	-0.67	-0.597	-0.616
R^2	0.604	0.611	0.623	0.633	0.562	0.605	0.598	0.615	0.61	0.609
p	0.129	0.092	0.057	0.09	0.728	0.092	0.091	0.05	0.113	0.082
PB										
Step 1										
β	-0.059	-0.059	-0.059	-0.059	-0.066	-0.079	-0.067	-0.059	-0.059	-0.059
R^2	0.004	0.004	0.004	0.004	0.004	0.006	0.004	0.004	0.004	0.004
p	0.713	0.713	0.713	0.713	0.687	0.634	0.686	0.713	0.713	0.713
Step 2										
β	0.053	0.005	0.022	0.013	0.016	-0.037	0.200	0.041	0.062	0.028
R^2	0.006	0.004	0.004	0.004	0.005	0.008	0.040	0.005	0.007	0.004
p	0.748	0.976	0.892	0.935	0.923	0.828	0.228	0.805	0.702	0.885
Step 3										
β	0.409	0.414	0.409	0.415	0.400	0.445	0.392	0.402	0.339	0.406
R^2	0.164	0.168	0.165	0.168	0.161	0.201	0.181	0.162	0.162	0.164
p	0.012*	0.010*	0.011*	0.010*	0.014*	0.006*	0.021*	0.012*	0.013*	0.012*
Step 4										
β	-0.311	-0.353	-0.319	-0.315	0.301	-0.306	-0.267	-0.413	-0.375	-0.333
R^2	0.175	0.178	0.177	0.208	0.183	0.174	0.169	0.182	0.181	0.176
p	0.498	0.457	0.533	0.593	0.78	0.48	0.59	0.35	0.457	0.47
NB										
Step 1										
β	0.175	0.175	0.175	0.175	0.166	0.178	0.189	0.175	0.175	0.175
R^2	0.031	0.031	0.031	0.031	0.028	0.032	0.036	0.031	0.031	0.031
p	0.273	0.273	0.273	0.273	0.305	0.278	0.249	0.273	0.273	0.273
Step 2										
β	0.012	-0.002	-0.020	-0.001	-0.047	-0.061	0.311	-0.051	0.001	-0.026
R^2	0.031	0.031	0.031	0.031	0.030	0.035	0.132	0.033	0.031	0.031
p	0.943	0.990	0.903	0.997	0.776	0.716	0.053	0.752	0.994	0.871
Step 3										
β	0.865	0.852	0.854	0.858	0.844	0.848	0.793	0.847	0.841	0.849
R^2	0.735	0.728	0.732	0.732	0.725	0.737	0.693	0.732	0.717	0.729
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 4										
β	-0.592	-0.634	-0.625	-0.765	0.11	-0.664	-0.849	-0.652	-0.573	-0.669
R^2	0.759	0.762	0.762	0.788	0.693	0.739	0.767	0.76	0.751	0.765
p	0.067	0.041*	0.042*	0.007*	0.906	<0.0001*	0.009*	0.03*	0.075	0.026*

Table 5.3. Hierarchical regression analysis of correlation between UPS gene expression and whole body protein kinetics.

PS	Atrogin1	MuRF1
Step 1		
β	0.048	0.038
R^2	0.002	0.001
p	0.764	0.816
Step 2		
β	0.021	0.065
R^2	0.003	0.006
p	0.896	0.699
Step 3		
β	0.783	0.783
R^2	0.583	0.573
p	<0.001*	<0.001*
Step 4		
β	-0.449	-0.543
R^2	0.597	0.6
p	0.26	0.138
PB		
Step 1		
β	-0.059	-0.066
R^2	0.004	0.004
p	0.713	0.687
Step 2		
β	0.032	0.065
R^2	0.005	0.008
p	0.846	0.697
Step 3		
β	0.413	0.405
R^2	0.166	0.161
p	0.011*	0.015*
Step 4		
β	-0.286	-0.246
R^2	0.174	0.168
p	0.543	0.596
NB		
Step 1		
β	0.175	0.166
R^2	0.031	0.028
p	0.273	0.305
Step 2		
β	-0.006	0.027
R^2	0.031	0.028
p	0.971	0.871
Step 3		
β	0.866	0.873
R^2	0.739	0.735
p	<0.001*	<0.001*
Step 4		
β	-0.425	-0.657
R^2	0.72	0.769
p	0.28	0.03*

Table 5.4. Hierarchical regression analysis of correlation between mitochondrial biogenesis gene expression and whole body protein kinetics.

PS	NRF1	PGC1α	PPARγ	Tfam	UCP2
Step 1					
β	0.048	0.048	-0.013	0.048	0.048
R^2	0.002	0.002	0.000	0.002	0.002
p	0.764	0.764	0.950	0.764	0.764
Step 2					
β	0.027	0.060	-0.347	-0.018	0.046
R^2	0.003	0.006	0.116	0.003	0.004
p	0.868	0.720	0.096	0.911	0.779
Step 3					
β	0.765	0.763	0.763	0.764	0.765
R^2	0.570	0.566	0.652	0.574	0.568
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 4					
β	-0.62	-0.398	-0.277	-0.62	-0.72
R^2	0.603	0.58	0.655	0.606	0.609
p	0.091	0.279	0.676	0.094	0.06
PB					
Step 1					
β	-0.059	-0.059	-0.098	-0.059	-0.059
R^2	0.004	0.004	0.010	0.004	0.004
p	0.713	0.713	0.632	0.713	0.713
Step 2					
β	0.044	0.108	-0.197	0.035	0.047
R^2	0.005	0.015	0.047	0.005	0.006
p	0.789	0.515	0.353	0.830	0.772
Step 3					
β	0.403	0.392	0.428	0.401	0.404
R^2	0.163	0.163	0.216	0.162	0.163
p	0.012*	0.015*	0.041*	0.012*	0.012*
Step 4					
β	-0.27	-0.015	0.123	-0.31	-0.37
R^2	0.17	0.163	0.221	0.173	0.175
p	0.581	0.907	0.708	0.493	0.477
NB					
Step 1					
β	0.175	0.175	0.118	0.175	0.175
R^2	0.031	0.031	0.014	0.031	0.031
p	0.273	0.273	0.566	0.273	0.273
Step 2					
β	-0.013	-0.045	-0.355	-0.085	0.017
R^2	0.031	0.033	0.135	0.038	0.031
p	0.938	0.786	0.085	0.599	0.915
Step 3					
β	0.847	0.858	0.788	0.846	0.844
R^2	0.725	0.741	0.707	0.739	0.718
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 4					
β	-0.78	-0.72	-0.687	-0.71	-0.82
R^2	0.772	0.779	0.749	0.773	0.767
p	0.009*	0.017*	0.074	0.026*	0.01*

Table 5.5. Hierarchical regression analysis of correlation between fat metabolism gene expression and whole body protein kinetics.

PS	Cpt1	FATP1	FATP4	PPARα	SREBP1	ACC
Step 1						
β	0.048	0.048	0.048	0.048	0.048	0.048
R^2	0.002	0.002	0.002	0.002	0.002	0.002
p	0.764	0.764	0.764	0.764	0.764	0.764
Step 2						
β	-0.232	0.002	-0.009	-0.021	0.000	-0.011
R^2	0.056	0.002	0.002	0.003	0.002	0.002
p	0.149	0.990	0.956	0.897	1.000	0.945
Step 3						
β	0.739	0.767	0.769	0.765	0.769	0.765
R^2	0.559	0.574	0.577	0.575	0.576	0.574
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 4						
β	0.268	-0.684	-0.655	-0.622	-0.661	-0.71
R^2	0.569	0.617	0.616	0.568	0.617	0.621
p	0.373	0.051	0.064	0.075	0.056	0.041*
PB						
Step 1						
β	-0.059	-0.059	-0.059	-0.059	-0.059	-0.059
R^2	0.004	0.004	0.004	0.004	0.004	0.004
p	0.713	0.713	0.713	0.713	0.713	0.713
Step 2						
β	-0.206	0.010	-0.010	0.006	0.023	-0.002
R^2	0.046	0.004	0.004	0.004	0.004	0.004
p	0.202	0.950	0.951	0.971	0.889	0.991
Step 3						
β	0.370	0.408	0.411	0.406	0.406	0.407
R^2	0.172	0.165	0.168	0.165	0.164	0.166
p	0.023*	0.011*	0.010*	0.011*	0.011*	0.011*
Step 4						
β	0.427	-0.453	-0.465	-0.382	-0.401	-0.49
R^2	0.203	0.167	0.19	0.181	0.183	0.191
p	0.241	0.332	0.332	0.403	0.372	0.296
NB						
Step 1						
β	0.175	0.175	0.175	0.175	0.175	0.175
R^2	0.031	0.031	0.031	0.031	0.031	0.031
p	0.273	0.273	0.273	0.273	0.273	0.273
Step 2						
β	-0.136	-0.011	-0.002	-0.049	-0.033	-0.018
R^2	0.049	0.031	0.031	0.033	0.032	0.031
p	0.394	0.945	0.989	0.766	0.838	0.91
Step 3						
β	0.846	0.843	0.841	0.842	0.849	0.839
R^2	0.708	0.721	0.718	0.727	0.730	0.719
p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Step 4						
β	-0.11	-0.623	-0.551	-0.61	-0.655	-0.62
R^2	0.713	0.752	0.743	0.756	0.764	0.75
p	0.428	0.041*	0.073	0.045*	0.03*	0.041*

Table 5.6. Sequences of primers used for real-time PCR.

Gene Name	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')
Autophagy		
P62	GAGCGGCTCTGGACACCAT	GTGGGCAAAAGTGGTCACAA
P53	TGCAATAGGTGTGCGTCAGAA	CCCCGGGACAAAGCAAA
ATF4	CAGACGGTGAACCCAATTGG	CAACCTGGTCGGGTTTTGTT
GADD45A	GATGTGGCTCTGCAGATCCA	ATGTCGTTCTCGCAGCAAAA
Beclin1	CAAGATCCTGGACCGTGTCA	CCTGGGCTGTGGTAAGTAATGG
LC3B	GGCGCTTACAGCTCAATGCT	TGCTGTGTCCGTTCACCAA
ULK1	AAAGCGAATTTTGTGTGATTTCC	CCCAACAATTCCAAAGGTTTATTT
ATG3	GGGCCGGCCGCTACT	CCAGTGCCTTTCCCTTCACA
ATG5	AAACCCATTCCTTCCAAGCTAGT	GCCAGGGACCACAGTGAAA
ATG7	AGCAGCCCACAGATGGAGTAG	ACGGTCACGGAAGCAAACA
UPS		
Atrogin1	AAGGTAGCGGGTGTGTATTATGC	TCATGGGAAAGGGTATGTGAATC
MuRF1	CAACCTGTGCCGGAAGTGT	CTGGTCCAGTAGGGATTTGCA
Mitochondrial Biogenesis		
PPARγ	GACCACTCCCACTCCTTTGA	GATGCAGGCTCCACTTTGAT
PGC1α	GGAACTGCAGGCCTAACTCC	CACTGTCCCTCAGTTCACCG
NRF1	CCACAGGCAGATGAATGTCTTG	TCCTGGGAAGGAGAGGAGATG
Tfam	ATGCTTATAGGGCGGAGTGG	TGGTTTCCTGTGCCTATCCA
UCP2	TCAGTGCTGGTGGAGTTGACA	GGGATCCTGGCTGGTACGA
Fat Metabolism		
PPARα	GACCACTCCCACTCCTTTGA	GATGCAGGCTCCACTTTGAT
ACC	CATTAGCACAGACATACCT	CACCAATACTCACTTCACT
SREBP1	GTATCAGGCAACTCACTAC	AACATCCATCACTCAACAG
FATP1	TCTTCTGGTCACTACTCA	CCTCGCTCTGTAATCATAA
FATP4	CCTGTTGTTCCTCTACTT	CCTGATGGTCTTGATGAA
Cpt1	TGAGCGACTGGTGGGAGGAG	GAGCCAGACCTTGAAGTAGCG
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

6 Chapter 5. Leucine decreases intramyocellular lipid deposition in an mTORC1-independent manner in palmitate-treated C2C12 myotubes

6.1 Abstract

Higher intramyocellular lipid (IMCL) deposition in skeletal muscle is commonly observed in patients with obesity, resulting in mitochondrial damage. Palmitic acid, a saturated fatty acid, has been reported to induce obesogenic conditions in C2C12 myotubes. Leucine has been shown to improve obesity-related metabolic signatures; however, evidence for the effect of leucine on IMCL and the underlying mechanisms are still lacking. The objective of this study was to determine the effect of leucine on IMCL deposition and identify the potential mechanisms. Palmitate-treated C2C12 myotubes were used as an *in vitro* model of obesity. Two doses of leucine were used: 0.5 mM (postprandial physiological plasma concentration) and 1.5 mM (supraphysiological plasma concentration). Rapamycin was used to determine the role of mammalian target of rapamycin complex 1 (mTORC1) in leucine's regulation of lipid deposition in C2C12 myotubes. One-way ANOVA followed by Tukey's post hoc test was used to calculate differences between treatment groups. Our results demonstrate that leucine reduces IMCL deposition in an mTORC1-independent fashion. Furthermore, leucine acts independently of mTORC1 to upregulate gene expression related to fatty acid metabolism and works through both mTORC1-dependent and mTORC1-independent pathways to regulate mitochondrial biogenesis in palmitate-treated C2C12 myotubes. In agreement with increased mitochondrial biogenesis, increased mitochondrial content, circularity, and decreased autophagy are observed in the presence of 1.5 mM leucine. Taken together, the results indicate leucine reduces IMCL potentially through an mTORC1-independent pathway in palmitate-treated C2C12 myotubes.

6.2 Introduction

Obesity continues to be a growing concern in the United States and around the world (17). According to the Centers for Disease Control and Prevention, 39.8% of adults and 18.5% of children are classified as obese (35a). Obesity is frequently accompanied by multiple chronic diseases, such as type 2 diabetes, cardiovascular diseases, and certain types of cancer. One of the most significant features of obesity is the overaccumulation of fat mass (6). Overaccumulation of fat mass with obesity increases the physical load placed on skeletal muscle, increasing the susceptibility of skeletal muscle to intramyocellular lipid (IMCL) deposition, which can result in physical damage, leading to decreased quality of life (45).

Skeletal muscle is a major site of metabolic activity and the most abundant tissue in the human body (39). For skeletal muscle to function at optimal levels, the efficient activation of processes that regulate muscle development, growth, regeneration, and metabolism is required (15). A loss or reduction in skeletal muscle function often leads to increased morbidity and mortality, either directly or indirectly, via the development of secondary diseases, such as diabetes, obesity, and cardiovascular disease. Mitochondria are particularly important for skeletal muscle function. The mitochondria play a critical role in fuel utilization and energy production and impact whole-body metabolic homeostasis (37). Mitochondrial dysfunction is frequently associated with ectopic lipid deposition, which may result in reduced insulin signaling and action and increased risk for development of obesity and other chronic diseases. Thus, improvement of mitochondrial function may decrease the amount of intracellular lipids and allow for a more adaptive response to chronic fuel excess, as with obesity or inactivity (37, 44).

Mitochondrial biogenesis refers to the generation of more mitochondria in response to either increased energy demands or to support cell growth and division. Mitochondrial

biogenesis is influenced by environmental stress, such as exercise, caloric restriction, and oxidative stress (20). Peroxisome proliferator- activated receptor-α-coactivator-1α (PGC1α), PGC1α, nuclear respiratory factors (NRFs) -1 and -2, and mitochondrial transcription factor A (Tfam) are major regulators of mitochondrial biogenesis. AMP-activated protein kinase regulates intracellular energy metabolism in response to acute energy crises and is a major regulator of mitochondrial biogenesis through PGC1α and the NRFs (20).

Mitochondria also play a key role in ensuring adequate levels of ATP needed for muscle contraction (37). In addition, the capacity of skeletal muscle to contribute to whole-body energy expenditure is related to the fact that muscle makes up ~40% of total body mass and accounts for 20%–30% of total resting oxygen uptake (47). This indicates that mitochondrial functional capacity is likely to directly affect muscle metabolic function and have a significant impact on whole-body metabolism (37).

At the molecular and structural level, mitochondrial biogenesis and function are reduced in patients with obesity (38). For example, when patients with obesity were compared with their lean counterparts, a lower mitochondrial content was observed in skeletal muscle (21). A comparative proteomic analysis examined subsarcolemmal and intermyofibrillar mitochondrial proteins from patients with obesity, and lean patients which found an increase in proteins related to electron transport chain complex II and citric acid cycle with obesity (24). Increased intramyocellular lipid content is associated with reduced mitochondrial phosphorylation activity in skeletal muscle (32). In skeletal muscle taken from women with obesity, the expression of carnitine palmitoyltransferase (Cpt) 1b, the rate-limiting enzyme responsible for fatty acid metabolism, is significantly downregulated (30) as well as peroxisome proliferator-activated

receptor γ (PPARγ), PGC1α, and NRF1, suggesting these genes may play a role in the dysregulation of muscle function associated with obesity (36).

The mammalian target of rapamycin complex 1 (mTORC1) is a kinase that regulates cell size, growth, and survival and is an important component of nutrient and energy pathways. Specifically, mitochondrial oxidative function is controlled by mTORC1 through a ying yang 1-PGC1 transcriptional complex (8). Ying yang 1 is a transcription factor and is a common target of both mTORC1 and PGC1α. In addition, humans and rodents treated with rapamycin (an mTORC1 inhibitor) display symptoms of diabetes, such as elevated blood triglycerides and cholesterol and reduced oxidative function in skeletal muscle (8). This suggests that an increase in mTORC1 signaling could result in improved mitochondrial oxidative function.

A crucial function of mTORC1 is the coupling of cellular nutrient sensing to metabolic homeostasis. The branched-chain amino acid leucine is an essential amino acid that serves as a potent activator of the mTORC1 signaling pathway, leading to activation of protein synthesis (2). Leucine has also been shown to improve adipocyte lipid metabolism, increase mitochondrial ATP production rate (46), and improve mitochondrial biogenesis in skeletal muscle (43). In addition, increasing dietary leucine has been shown to decrease diet-induced obesity, hypercholesterolemia, and hyperglycemia in mice fed a high-fat diet (29, 51). More recently, it has been shown that increasing leucine concentrations in C2C12 myotubes can decrease fat uptake into cells through an increase in fat oxidation (11), increase expression of genes related to mitochondrial biogenesis (41), and increase mitochondrial density (48). Taken together, these data suggest that leucine has the potential to regulate IMCL by increasing mitochondrial biogenesis.

Therefore, the objective of this study was to determine the effect of leucine supplementation on IMCL deposition under obese conditions and identify potential mechanisms of action using an *in vitro* model. We hypothesized that leucine would decrease IMCL in C2C12 myotubes through activation of the mTORC1 pathway, potentially through increased mitochondrial function.

6.3 Materials and Methods

6.3.1 Cell culture and experimental design

C2C12 mouse myoblasts were obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA) containing 25 mmol/L glucose, 10% FBS (Gibco, Berlin, Germany), and antibiotics (1% penicillin-streptomycin) at 37°C in 5% CO2. When the number of cells reached 70%–80% confluence, cells (passage < 12) were seeded into 6-well plates for experimentation. Differentiation of C2C12 cells was induced using DMEM containing 2% horse serum and 1% antibiotics. Every well was seeded with 100,000 cells. Cells were then incubated in the differentiation media for 4 days, and media was changed every 24 h. For all treatments, cells were seeded in triplicate and then each experiment was repeated three times, yielding nine wells per treatment. All differentiated cells were divided into two groups: palmitate (PA) treated and control. Furthermore, each group was assigned the following treatments: control (no treatment), rapamycin, 0.5 mM leucine, 1.5 mM leucine, 0.5 mM leucine + rapamycin, and 1.5 mM leucine + rapamycin.

6.3.2 Preparation of palmitic acid-BSA solution

Palmitic acid was conjugated with fatty acid-free BSA according to Chavez et al. (5). Briefly, sodium palmitic acid (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol at

90°C and conjugated with 2% fatty acid-free BSA, which was prepared in a customized Dulbecco's modified Eagle's medium that contains no L-leucine (leucine-free DMEM; Athena Enzyme System, Baltimore, MD) and incubated in an isothermal water bath at 37°C. This was the stock solution of palmitic acid (PA) at a concentration of 4 mM. Stock palmitic acid solution was diluted with leucine-free DMEM to 0.75 mM for a final working solution (26). The dose of palmitate (PA) used in this study was adapted from a previously published study by Li et al. (26). In addition, recent research demonstrates that cell death starts to manifest in C2C12 myotubes treated with PA over 24 h (4). To determine optimal treatment time with PA, we conducted a pilot study using 6, 12, and 24-h incubations with PA. Oil red O stain showed that C2C12 myotubes treated with PA for 24 h had the highest percentage of intracellular lipid droplet deposition with no change in cell death compared with the 6 and 12-h time points (Figure 5.7). Therefore, 24 h PA treatment was selected for later experiments to induce maximum intracellular fatty acid deposition.

6.3.3 Leucine and rapamycin treatment

L-leucine (EMD Millipore Corp., Billerica, MA) was dissolved in ultrapure water as stock concentration of 0.125 M. It is well established that leucine activates mTORC1. To determine optimal mTORC1 activation by leucine, dose-response and time course experiments were conducted. The efficacy of leucine was determined by measuring phosphorylation levels of proteins downstream of mTORC1, 4E binding protein 1 (4EBP1), and p70S6K1. All cells were grown in leucine-free media for either 6, 12, or 24 h. Cells were treated with two doses of leucine to give a final concentration of 0.5 mM (postprandial physiological plasma concentration) and 1.5 mM (supraphysiological plasma concentration) leucine. All starvation times of leucine deprivation reduced activation of translation initiation downstream of mTORC1

(e.g., phosphorylation of 4EBP1 and p70S6K), which were recovered by both doses of leucine (Supplemental Fig. S2). Additionally, the role of mTORC1 in leucine activation of translation initiation was confirmed by using rapamycin, an inhibitor of mTORC1 (Figure 5.8). Thus, both leucine doses were chosen for later experiments. In experiments involving rapamycin (EMD Chemicals, San Diego, CA), 100 nM of rapamycin was added to media 1 h before treatments with PA and/or leucine.

6.3.4 RNA isolation and real-time PCR

RNA samples were isolated using TRIzol (Invitrogen, Waltham, MA), following manufacturer instructions. cDNA was synthesized in accordance with manufacturer instructions (Roche Lightcycler 480 system). SYBR green master mix (Quanta, Gaithersburg, MD) was used as the reporter dye for acetyl- CoA carboxylase (ACC), Cpt1a, peroxisome proliferator-activated receptor γ (PPARγ), fatty acid transport protein 1 (FATP1), fatty acid transport protein 4 (FATP4), sterol regulatory element-binding protein 1 (SREBP1), adenine nucleotide translocase type 1 (ANT1), PPAR, mitochondrial transcription factor A (Tfam), uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3), nuclear respiratory factor 1 (NRF1) and sirtuin 1 (SIRT1). All primer sequences are provided in the supplementary material (Table 5.1). Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene to normalize data since it remains constant regardless of treatment. All primers were ordered from Integrated DNA Technologies (Coralville, IA). All samples and controls were analyzed in duplicate. Relative expression of target genes were determined using $2^{\Delta\Delta Ct}$ method (28).

6.3.5 Oil red O staining and imaging

Oil red O staining was performed following the manufacturer's instructions (American MasterTech, Lodi, CA). Briefly, DMEM was removed from cells and the cells were washed with

PBS. PBS was removed from each well and propylene glycol was added and incubated for 2 min. Then, propylene glycol was removed, cells were incubated with oil red O stain, which was preheated in 60°C water bath, for 6 min. Oil red O stain was removed, and cells were incubated with 85% propylene glycol for 1 min. After rinsing with ultrapure water three times, modified Mayer's hematoxylin was used for 1 min. Cells were rinsed again with ultrapure water three times. Finally, cells were dried at room temperature, and 10% formalin was added to fix cells. Aqueous mounting solution was added and coverslips were placed in each well. Images were obtained using an inverted microscope (VWR, Radnor, PA) at 40 × magnification. All cells were 90% differentiated before treatment was administered. However, it has been shown that rapamycin may impact C2C12 hypertrophy (14), which could impact images. Images were processed using ImageJ software (National Institutes of Health, Bethesda, MD). The percentage of red stain in each image was exported from ImageJ (National Institutes of Health) and analyzed using GraphPad Prism Version 6.0 (La Jolla, CA).

6.3.6 Cell lysate sample collection and Western blotting

Myotubes were cultured in 35-mm-diameter 6-well plates. After treatment, cell culture media was removed and cells were washed once with PBS (VWR, Atlanta, GA). Then 1 × Laemmli sample buffer containing 65.8 mM Tris-HCl (pH 6.8), 26.3% (wt/vol) glycerol, 2.1% SDS, and 0.01% bromophenol blue with 1% (vol/vol) protein inhibitor cocktail (VWR, Center Valley, PA) was added before sample collection. Cell lysate samples were collected and incubated at 100°C for physical denaturation. Cell lysate samples were then vortexed and centrifuged at 4°C, 12,000 revolutions/min. All primary and secondary antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Relative protein content of eukaryotic translation initiation factor 4E binding protein 1 (4EBP1; catalog no. 9644S),

phospho-4EBP1 (p-S65; catalog no. 9456S), p70 ribosomal S6 kinase 1 (p70S6K1; catalog no. 2708S), phosphop70S6K1 (p-T389; catalog no. 9234S), mitochondrial dynamin-like GTPase (Opa1; catalog no. 67589S), microtubule-associated proteins 1A/1B light chain 3B (LC3-II; catalog no. 3868S), mTORC1 (catalog no. 2983S), phospho-mTOR (p-S2448; catalog no. 5536S), p62/SQSTM1 (catalog no. 5114S), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalog no. 5174S) were examined. All primary antibodies were diluted as the ratio 1:2,500 (vol/vol), and all secondary antibodies were diluted as the ratio 1:5,000 (vol/vol). Blots were developed with enhanced chemiluminescence (VWR). Relative protein content was analyzed using a ProteinSimple FluorChem M imager (ProteinSimple, San Jose, CA) and band gray values were quantified using ImageJ software. Ponceau S staining was used as a loading control. GAPDH was used to normalize Opa1, LC3-II, and p62. Phosphorylated 4EBP1, mTORC1, and p70S6K1 were normalized to the total protein content of 4EBP1, mTOR, and p70S6K1, respectively. Finally, all treatments were expressed as the fold change compared with control.

6.3.7 Mitotracker imaging

Mitotracker Red CMXRos (Cell Signaling Technology) lyophilized solid was dissolved in 941 μL of DMSO to make a 0.1 mM stock solution. A 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Cell Signaling Technology) lyophilized solid was dissolved in ultrapure water to reach 20 mg/mL stock. Cells were cultured in a specialized 35-mm fluorodish (World Precision Instruments, Sarasota, FL) for immunofluorescence. After treatment, mitotracker red probes were directly added into cell culture medium to reach a final concentration of 100 nM and incubated 30 min at 37°C. After incubation, cells were washed with twice with PBS and incubated with PBS containing 0.5 g/mL DAPI. Then, cells were rinsed once in PBS and then

fixed with 3.7% formalin for imaging. For cell imaging, an inverted Nikon Ti-E Eclipse Total Internal Reflection Fluorescence Microscope equipped with × 100 1.49 numerical apertures oil immersion Apo Nikon objective, Zyla 4.2 sCMOS Camera (Andor Technology, Belfast, Northern Ireland), and AURA II solid state triggered illuminator with a 4-channel light source (395 nm, 485 nm, 560 nm, 640 nm) was used. Images (n = 9 per treatment) were analyzed using the "Mito-Morphology" macro for ImageJ software specifically designed for mitochondrial measurements (10).

6.3.8 Statistical analysis

Summary statistics were calculated for all data. One-way ANOVA followed by Tukey's post hoc test was used to calculate differences between treatment groups. Results are reported as mean ± SE. All data analysis was performed using Prism Graph-Pad Software Version 6.0 (La Jolla, CA). $P < 0.05$ was considered statistically significant.

6.4 Results

Leucine is well known for activating the mTORC1 pathway in multiple cell types (16, 19), leading to activation of the translation initiation factors, which can increase protein synthesis. In Figure 6.1, the ability of leucine to activate translation initiation through an mTORC1-dependent manner was validated.

Both 0.5 mM and 1.5 mM leucine increased phosphorylation of 4EBP1 ($P < 0.0001$) in control C2C12 cells, and the 1.5 mM leucine dose increased phosphorylation of 4EBP1 in PA-treated cells. Leucine increased phosphorylation of p70S6K1 in PA-treated cells ($P < 0.0001$)

and mTORC1 phosphorylation ($P < 0.0001$) in control cells. Rapamycin inhibited the effect of leucine in both control and PA-treated cells.

In Figure 6.2, oil red O staining demonstrates that leucine reduces intracellular lipid droplet deposition in PA-treated C2C12 myotubes, independently of mTORC1. However, in this study, leucine acts independently of mTORC1 to inhibit PA deposition. In the absence of PA, there was no effect of either leucine or rapamycin.

Kelley et al. (21) first demonstrated that the skeletal muscle from individuals with obesity has impaired mitochondrial respiratory chain enzyme activity and damaged inner mitochondrial membrane morphology. In addition, Ritov et al. (38) reported reduced overall electron transport chain enzyme activity in the vastus lateralis muscle from both patients with obesity and patients with type 2 diabetes, which was accompanied by a decrease in the relative number of mitochondria compared with lean counterparts. Therefore, we wanted to determine whether 1) the function or biogenesis of mitochondria is affected by PA treatment in C2C12 myotubes; 2) leucine treatment can reverse the effects of PA; and 3) the effects of leucine on PA-treated myotubes are mediated by mTORC1 pathway. Treatment with leucine increased expression of UCP2 (Figure 6.3A), ANT1 (Figure 6.3C), PGC1α (Figure 6.3D), PPARγ (Figure 6.3E), SIRT1 (Figure 6.3F), NRF1 (Figure 6.3G), and Tfam (Figure 6.3H) in control cells (Figure 6.3). However, 0.5 mM leucine did not increase PGC1α or Tfam expression and 1.5 mM leucine did not increase gene expression of UCP2 or ANT1 in control cells. There was no effect of leucine on UCP3 (Figure 6.3B), with the exception of 0.5 mM leucine in the presence of rapamycin, which increased UCP3 expression. Treatment with rapamycin blunted the effect of leucine on expression of ANT1, PPARγ, SIRT1, and Tfam in control cells, suggesting leucine mediates expression of some mitochondrial biogenesis in an mTORC1-dependent fashion. There was no

effect of rapamycin only on PGC1α compared with control cells; however, the rapamycin, in the presence of 0.5 mM leucine, altered the leucine response, resulting in an increase ($P < 0.05$) in PGC1α expression. There was no effect of rapamycin only on NRF1 in the presence of leucine.

Treatment with PA blunted the effect of leucine on UCP2, UCP3, ANT1, PGC1α (1.5 mM leucine only), and SIRT1 compared with control cells. In the presence of rapamycin, there was a decrease in ANT1, PPARγ, SIRT1, NRF1, and Tfam expression in both PA-treated cells and cells treated with PA and leucine. Taken together, these data suggest that leucine regulates mitochondrial biogenesis gene expression through mTORC1-independent and mTORC1-dependent pathways.

Although changes in mitochondrial biogenesis gene expression were observed, mitotracker staining (Figure 6.4A) revealed that the effect on mitochondrial content (Figure 6.4B), circularity (Figure 6.4C), and area (Figure 6.4D) was not as prevalent. In PA-treated cells, treatment with 1.5 mM leucine increased mitochondrial content, average circularity, and area. This effect was blunted by rapamycin; however, rapamycin treatment alone had no effect on PA-treated cells. Opa1 is reported to be a biomarker for indicating mitochondrial fusion (22). Our data show that Opa1 was increased in the presence of rapamycin in control cells, which was further increased by treatment with 1.5 mM leucine ($P < 0.001$; Figure 6.5B). Opa1 was increased with PA treatment compared with control (via t test between controls; $P < 0.0001$), and leucine increased Opa1 in PA-treated cells ($P < 0.01$; Figure 6.5D). Rapamycin downregulated Opa1 and blunted the effect of leucine in PA-treated cells ($P < 0.001$).

Next, we examined the involvement of autophagy in leucine- mediated increase in mitochondrial biogenesis and fusion. An important indicator of autophagosome machinery assembly is LC3-II (Figure 6.5C). There was no effect of 0.5 mM leucine on LC3-II under

control or PA treatment conditions with or without rapamycin. However, treatment with rapamycin only increased LC3-II in control cells ($P < 0.0001$). Treatment with rapamycin and 1.5 mM leucine increased LC3-II in both control and PA treatment conditions ($P < 0.05$) compared with treatment with 1.5 mM leucine in control and PA-treated conditions in the absence of rapamycin.

Under control conditions (with and without rapamycin), 1.5 mM leucine increased p62 ($P < 0.05$). Rapamycin-only treatment also increased p62 compared with control cells ($P < 0.05$). There was no effect of PA treatment on p62 compared with control cells; however, p62 was decreased with PA and rapamycin compared with rapamycin only. Both 0.5 and 1.5 mM increased p62 in PA-treated cells compared with PA and PA with rapamycin ($P < 0.01$). Rapamycin reduced the effect of 0.5 and 1.5 mM leucine on p62 in PA-treated cells ($P < 0.05$).

As part of the natural cell survival process, damaged organelles are recycled into the autophagosome-lysosome system (33). Increased mitophagy (autophagy of mitochondria) would explain observed decreased functional mitochondria in palmitate- treated myotubes (Figure 6.4, A and B), suggesting mitochondrial fusion may be involved in eliminating impaired mitochondria by fusing them together and producing healthy mitochondria (reviewed in Ref. 31). Together with mitotracker data, these data suggest that palmitate decreases the number of healthy mitochondria and mitochondrial fusion possibly through activation of mitophagy pathways. These data also suggest that leucine promotes mitochondrial fusion to protect mitochondria from palmitate-induced mitophagy through mTORC1 pathway.

Since treatment with leucine is able to decrease intracellular fatty acid deposition, a process that is inhibited by rapamycin, we assumed that fatty acid metabolism was involved in the effect of leucine on fatty acid deposition and that the mTORC1 pathway functions as the

upstream regulator of fatty acid metabolism. We measured gene expression related to fatty acid oxidation (Cpt1b), fatty acid synthesis (ACC, SREBP1), fatty acid transport (FATP1 and FATP4), and fatty acid gene transcription factor (PPARα).

Treatment with PA decreased gene expression in ACC, PPARα, FATP1, FATP4, and SREBP1 compared with control cells (Figure 6.6). PA increased Cpt1b (Figure 6.6A) expression compared with control cells. Both 0.5 and 1.5 mM leucine significantly increased gene expression of Cpt1b, ACC (Figure 6.6B), FATP1 (Figure 6.6D), FATP4 (Figure 6.6E), and SREBP1 in both control and PA-treated cells. Only 1.5 mM leucine increased PPARα (Figure 6.6C) in control cells, and both 0.5 mM and 1.5 mM leucine increased PPARα in PA-treated cells. In control cells, rapamycin-only increased expression of Cpt1b and decreased expression of ACC PPARα, FATP4, and SREBP1 (Figure 6.6F) and the presence of rapamycin blunted the effect of leucine on Cpt1b, PPARα, FATP1, FATP4 (1.5 mM leucine, only), and SREBP1 (1.5 mM leucine, only) under control conditions. Expression of ACC, FATP4, and SREBP1 in control cells increased with rapamycin and 0.5 mM leucine. In the presence of PA only, Cpt1b was increased compared with control cells. However, expression of ACC, PPARα, FATP1, FATP4, and SREBP1 was decreased in PA only compared with control cells. In PA and rapamycin-treated cells, there was an increase in Cpt1b, ACC, PPARα, FATP4, and SREBP1 compared with PA alone. There was no effect of leucine on Cpt1b with PA and rapamycin treatment, and ACC expression was decreased in cells treated with leucine, PA, and rapamycin. Although treatment with 0.5 and 1.5 mM leucine increased expression of PPAR, FATP1, and SREBP1 compared with PA and rapamycin, the effect of leucine was lower when compared with PA treatment with leucine in the absence of rapamycin.

These data suggest that fatty acid metabolism could be dysregulated under palmitate treatment, leading to increased intracellular fatty acid accumulation, and that leucine could promote fatty acid metabolism by upregulating fatty acid synthesis, fatty acid oxidation, and fatty acid transport to eliminate accumulated fatty acids through mTORC1. However, these data also demonstrate that leucine acts in an mTORC1- independent manner to regulate lipid deposition into muscle cells.

6.5 Discussion

To our knowledge, the present study is the first to demonstrate that the branched-chain amino acid leucine regulates IMCL deposition independently of mTORC1. Instead, inhibition of IMCL deposition occurs through regulation of mitochondrial biogenesis and fatty acid oxidation. These findings are supported by previous work in our laboratory (3, 12) in which we demonstrated that 42 days of leucine supplementation increased PPARγ expression in the skeletal muscle of male Sprague-Dawley rats (3). We also demonstrated that diets high in protein and leucine decrease IMCL, potentially through downregulation of mTORC1 in male Zucker rats (12).

High-fat feeding has been established as a model to induce obesity in animal models. However, there is no well-established model for simulating obesity in cultured muscle cells. One of the key differentiating factors in skeletal muscle between animal and human models of normal weight and those that are obese is the higher level of IMCL observed (1, 6). Moreover, levels of saturated fatty acids, especially the C16:0 fatty acid, palmitate, are higher in the plasma and skeletal muscle of individuals with obesity (40). In recent years, treating C2C12 myotubes with palmitate has been shown to partially mimic *in vivo* obese skeletal muscle conditions, such as increased IMCL deposition (49) and insulin resistance (34). Here, we validate these findings by

demonstrating that palmitate- treated C2C12 myotubes had almost sevenfold higher IMCL content compared with controls, which is similar to observations made in muscle from animal models of obesity (1), further establishing this as a viable *in vitro* model for studying the effects of obesity on skeletal muscle.

Since mitochondria are the primary site for fatty acid beta-oxidation, it has been suggested that mitochondrial dysfunction could lead to elevated IMCL deposition in skeletal muscle. In the *in vivo* model reported by Koves and colleagues (23), disordered mitochondrial function, including excessive beta-oxidation, is often observed in high fat-fed animals. In support of these results, we observed upregulated expression of genes targeting fatty acid oxidation in PA-treated cells, accompanied by decreased mitochondrial content in PA-treated cells. This aligns with data from studies using obese skeletal muscle, in which elevated expression of genes regulating fatty acid oxidation was observed compared with normal-weight counterparts (50).

The mechanisms related to observed dysfunctional mitochondria in obese skeletal muscle are complicated. One explanation for the dysfunction could be through increased autophagy signals in mitochondria, also known as mitophagy, which occurs in skeletal muscle. As the primary system for recycling damaged subcellular organelles, mitophagy enables myotubes to recycle any potential energy from impaired mitochondria. However, under obese conditions, dysregulated mitophagy could cause harm to the intramyocellular mitochondrial pool. However, markers of autophagy, LC3-II and p62, do not change in the skeletal muscle from individuals with obesity (25), which is similar to what we observed in this study (e.g., no change in the LC3-II protein content in the presence of PA).

It is well established that leucine initiates protein synthesis through activation of the mTORC1 pathway in skeletal muscle (7), which we further confirmed in this study. What is not

well known is the role of leucine in muscle mitochondria metabolism, especially under obese conditions. Sato et al. (41) recently administered leucine to both young rats and C2C12 myotubes. Administration of leucine upregulated genes related to mitochondrial biogenesis, Tfam and PGC1α, in the skeletal muscle of rats and in the C2C12 myotubes. Similar changes were observed in this study. This study also demonstrates the ability of leucine to increase expression of genes related to mitochondrial biogenesis, such as PGC1α and SIRT1, in C2C12 myotubes under control conditions and in cells treated with PA. Interestingly, gene expression under control conditions is not altered in the presence of rapamycin. However, when cells are treated with PA, rapamycin downregulates the effect of leucine on genes related to mitochondrial biogenesis, suggesting mTORC1 plays a role. These findings were translated into an increase in mitochondrial density in the C2C12 myotubes treated with PA and leucine, an effect that was also blunted with rapamycin. This is supported by work by Vaughan et al. (48), who found that leucine treatment of C2C12 cells increases mitochondrial density. These data indicate that the effect of leucine on mitochondrial biogenesis may to be regulated through mTORC1 under obese conditions.

Despite the increase in mitochondrial density and expression of genes related to mitochondrial biogenesis under obese conditions and the potential regulation by mTORC1, mTORC1 did play a role in the prevention of lipid deposition in skeletal muscle cells in the presence of leucine. Treatment with leucine significantly reduced lipid deposition in C2C12 myotubes. This could be partially explained by an increase in mitochondrial biogenesis; however, Estrada-Alcade et al. (11) demonstrate that in C2C12 myotubes, leucine increased lipid oxidation and reduced lipid incorporation.

Eight months of leucine supplementation in an *in vivo* model of obesity increased energy expenditure and were accompanied by significant elevations in gene expression related to mitochondrial oxidative function for genes, such as UCP3, PPARα, and NRF1 (16). However, some data suggest that dietary leucine supplementation does not change energy homeostasis, protein content of UCPs in adipose and muscle, or body composition (35). Notably, in these studies, leucine was only supplemented in a low-fat (5% wt/wt) diet, which means high-fat diet-induced obesity was not achieved. In another short-term (1-wk) study conducted by the same group using a high-fat diet-induced animal model of obesity, the leucine-supplemented group demonstrated decreased body weight gain, which could be explained by lower fat mass gain, food intake, and energy intake (13). At the transcriptional level, fatty acid synthesis-related genes, such as ACC, were downregulated in the liver of the leucine-supplemented group (13).

The current study has contradictory findings, since we observed upregulation of all genes related to fat metabolism in the presence of leucine. Another study showed increased body weight, partially because of higher adipose tissue gain, in a chronic leucine-supplemented (24 wk) diet-induced obese animal model, whereas insulin sensitivity was improved (27). Moreover, leucine supplementation in the diet of offspring from rats fed a high-fat diet, showed higher Cpt1 and PGC1α expression in the skeletal muscle, suggesting leucine improves fatty acid oxidation in muscle, which is similar to what we found in the present study. In another *in vivo* animal study, leucine supplementation after diet-induced obesity did not change gene expression related to fatty acid metabolism and mitochondrial biogenesis, such as Cpt1 and NRF1 in muscle, but did increase expression of genes, such as UCP1, UCP3, and Cpt1 in epididymal white adipose tissue and Cox-III, PPARα, UCP3, and Cpt1 in brown adipose tissue. Long-term leucine supplementation in myotubes has been reported to facilitate mitochondrial biogenesis (42).

Previous *in vivo* research from our laboratory also showed increased expression of markers such as FAS and PPARγ in skeletal muscle from obese Zucker rats fed with a high-protein diet containing a high amount of leucine (12). Moreover, treatment of C2C12 myotubes with palmitate and leucine reduced the effect of leucine on regulation of Tfam and PGC1α (19). Here, we report upregulation of mitochondrial biogenesis-related gene expression (e.g., PGC1α, PPARγ, NRF1, and Tfam) and mitochondrial content observed in palmitate-treated myotubes with leucine supplementation. However, the effect of leucine on obesity and skeletal muscle lipid deposition is inconsistent in the literature and the results are influenced by both the model of obesity (diet-induced obesity vs. genetically modified obese models) and the method of leucine administration (*in vivo* vs. *in vitro*).

There are several limitations to this study. First, this is an *in vitro* model of obesity in skeletal muscle using a murine cell line. Although we observed similar morphological changes to the *in vitro* muscle cells as has been observed in muscle from patients with obesity, we are working in an isolated cell system without the checks and balances that occur in the presence of whole-body systems. Another limitation is that our control cells were void of leucine, which is not physiological. Finally, tracer experiments to determine protein synthesis or lipid oxidation rates and bioenergetic studies were not performed in this study, which would have allowed us to identify physiological outcomes to the mechanistic changes we observed.

In conclusion, the present study is the first to demonstrate the role of leucine in intracellular fatty acid content and the related mechanisms of action in C2C12 muscle cells independently of mTORC1. The decrease in IMCL could be explained by an increased number of healthy mitochondria, which is supported by the increased mitochondrial content. This study

also suggests the possible involvement of fatty acid metabolism-related enzymes in leucine-mediated IMCL reduction. Additional studies are needed to confirm mechanisms of action.

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6.7 Figures

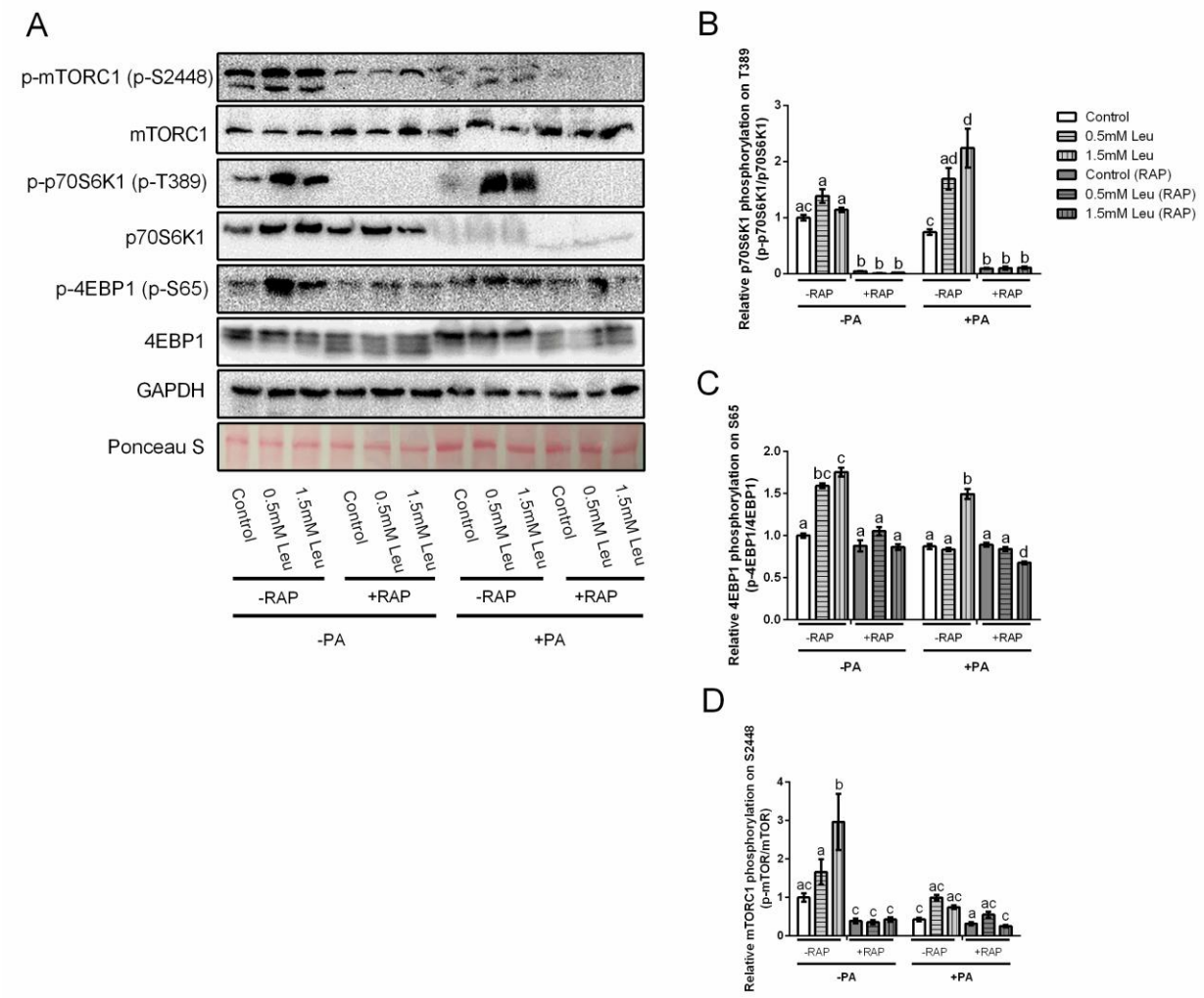

Figure 6.1. Leucine decreases the intracellular lipid deposition in palmitate (PA)-treated C2C12 myotubes. C2C12 myotubes were treated with or without PA. Both groups received six treatments: control, rapamycin (RAP), 0.5 mM leucine (0.5 mM Leu), 1.5 mM leucine (1.5 mM Leu), 0.5 mM leucine + rapamycin (0.5 mM Leu + RAP), and 1.5 mM leucine + rapamycin (1.5 mM Leu + RAP). A: oil red O staining of all treatments. B: quantification of oil red O staining. Values not sharing the same letter are significantly different; $P < 0.05$. Values represent mean ± SE (n = 9).

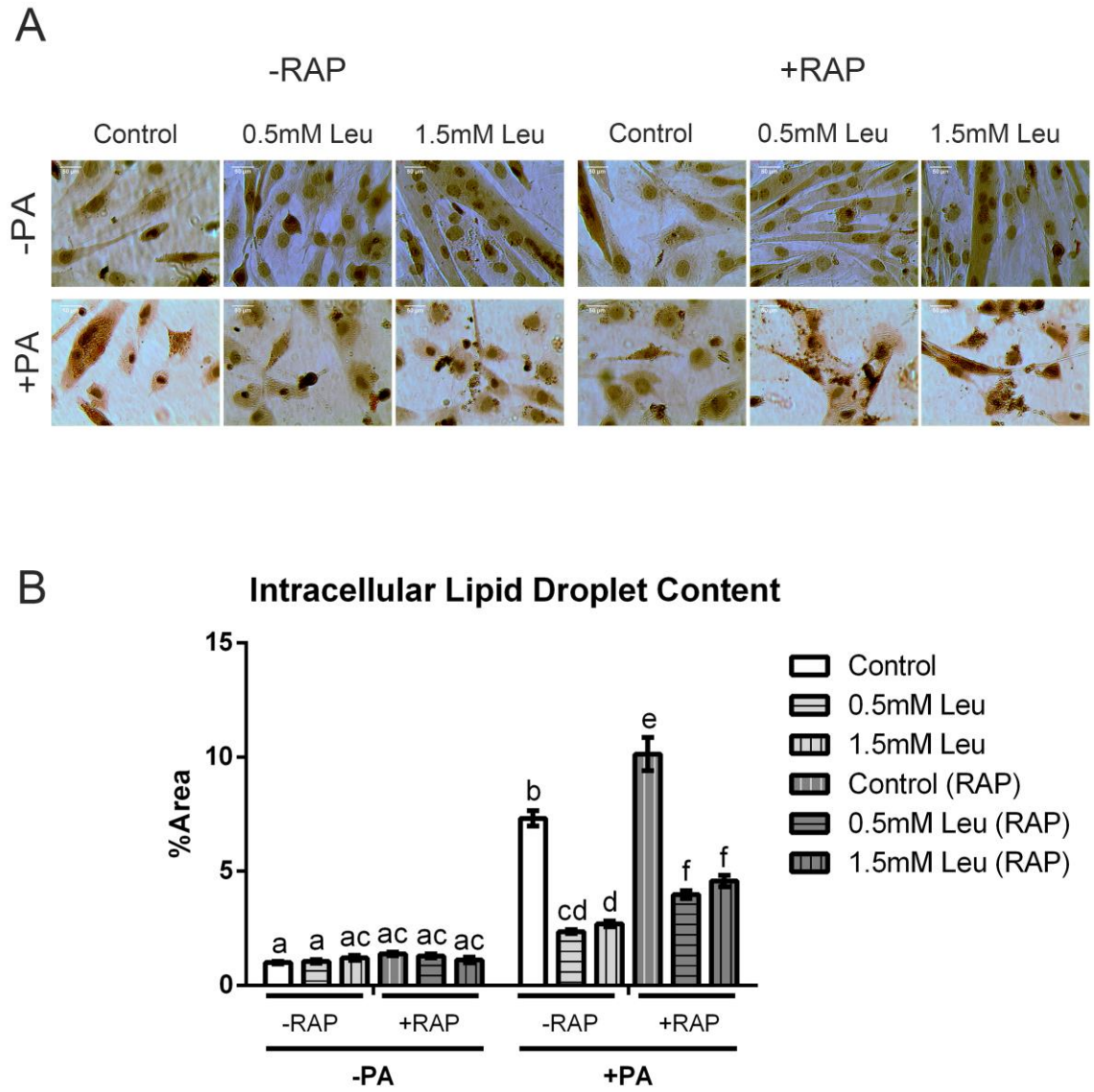

Figure 6.2. Leucine (Leu) activates translation initiation via the mammalian target of rapamycin (RAP) complex (mTORC) 1 pathway in C2C12 myotubes treated with or without palmitate (PA). Representative Western blot analysis (A) was performed to determine the phosphorylation of mTORC1 on S2448, total mTORC1, phosphorylation (p) of 4E binding protein 1 (4EBP1) on S65, total 4EBP1, phosphorylation of p70S6K1 on T389, total p70S6K1, and GAPDH. The PVDF membrane was stained by Ponceau S stain to ensure consistency in loading the gel. The relative phosphorylation level of 4EBP1 (B), p70S6K1 (C), and mTORC1 (D) were expressed as the fold-change to control group and ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P < 0.05$. Values represent mean ± SE ($n = 9$).

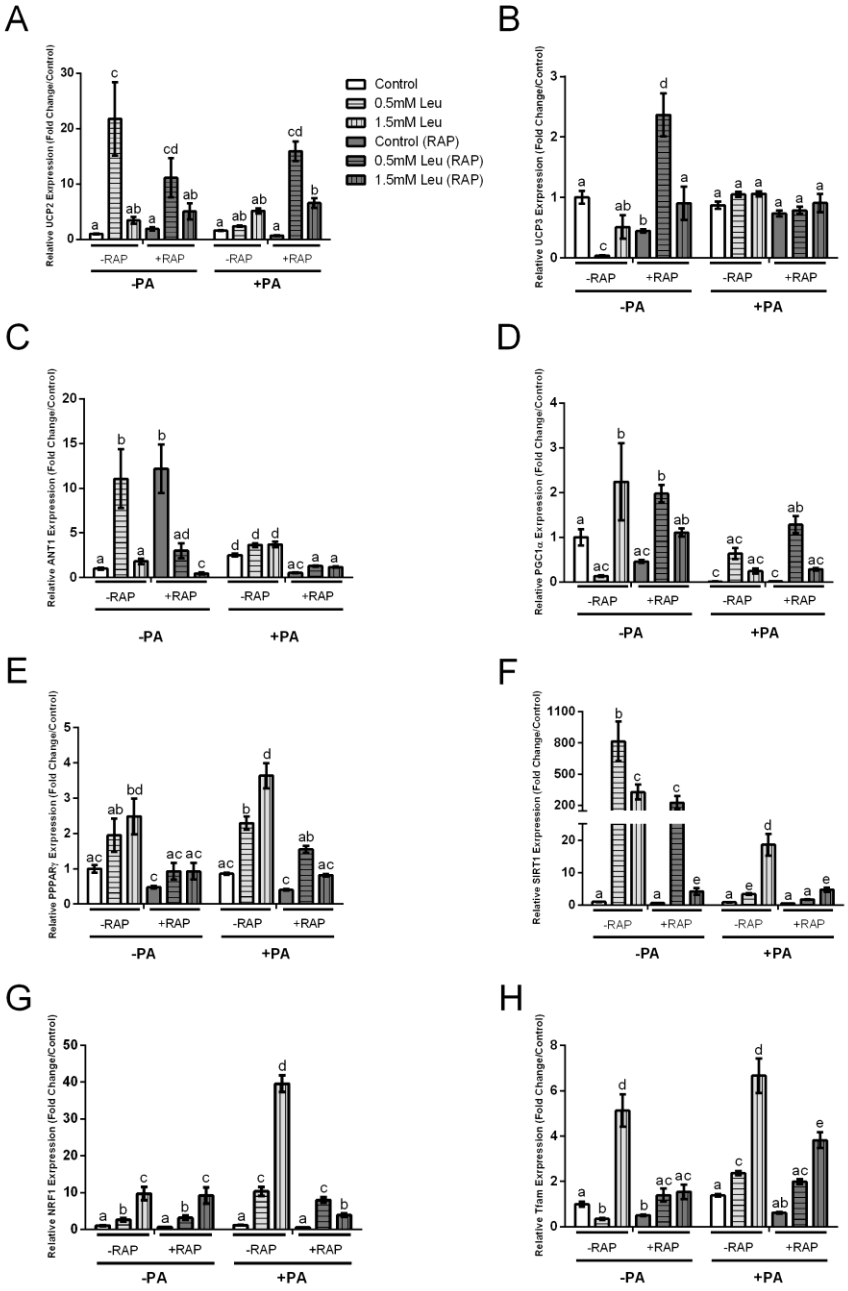

Figure 6.3. The effect of leucine (Leu) on expression of genes related to mitochondrial biogenesis in palmitate (PA)-treated C2C12 myotubes. Gene expression related to mitochondrial electron transport chain uncoupling: uncoupling protein (UCP) 2 (A), UCP3 (B), and adenine nucleotide translocase type 1 (ANT1) (C). Gene expression related to mitochondrial biogenesis: peroxisome proliferator-activated receptor-γ-coactivator-1α (PGC1α) (D), peroxisome proliferator-activated receptor γ (PPARγ) (E), sirtuin 1 (SIRT1) (F), nuclear respiratory factor (NRF) 1 (G), and mitochondrial transcription factor A (Tfam) (H). Values not sharing the same letter are significantly different; $P < 0.05$. Values represent mean ± SE (n = 9). RAP, rapamycin.

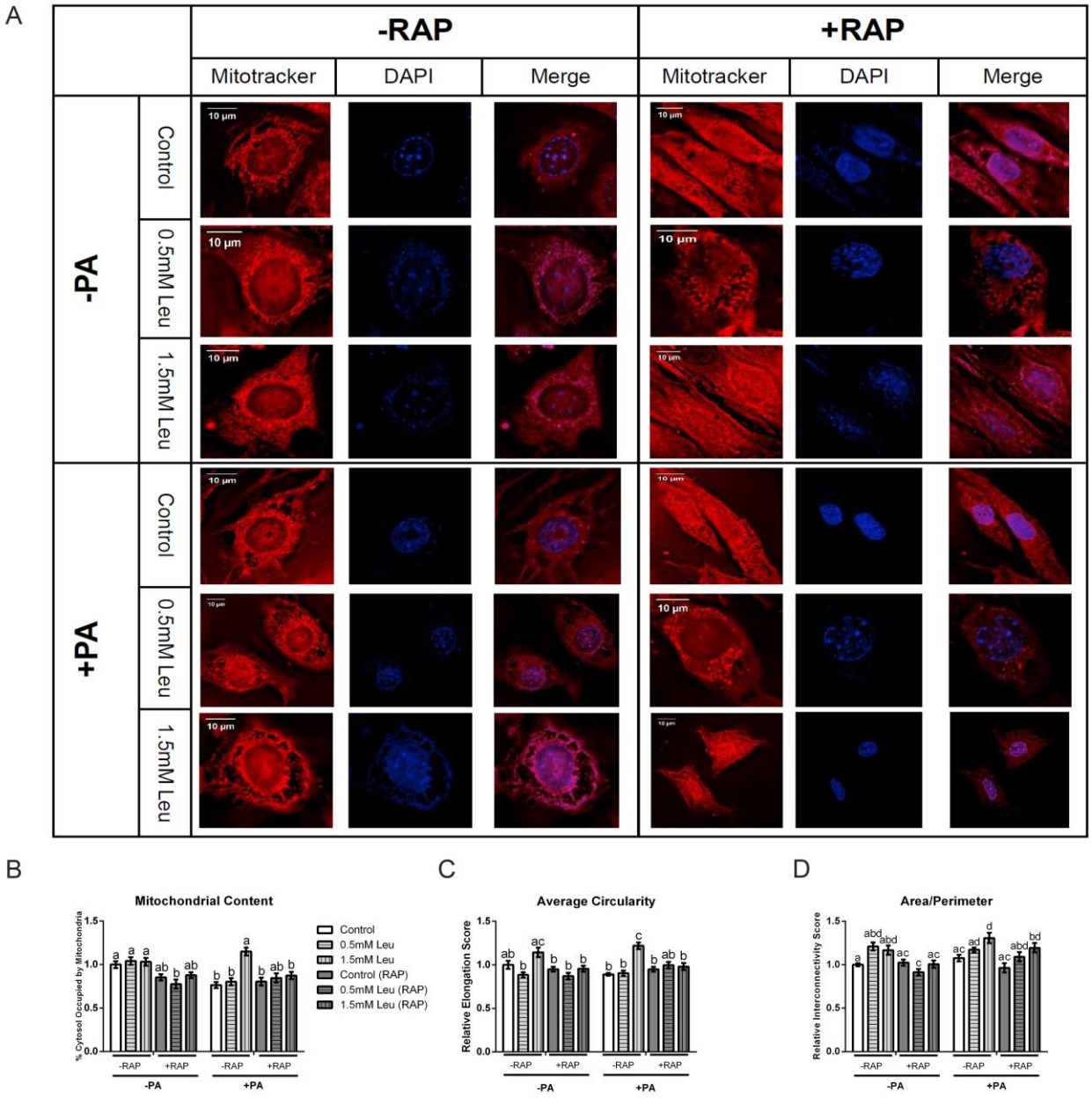

Figure 6.4. The impact of leucine (Leu) on mitochondrial content, circularity, and area in palmitate (PA)-treated C2C12 myotubes. A: mitotracker, DAPI, and merged staining of all treatment groups. Images were taken under ×100 magnification. B: quantification of percentage of cytosol occupied by mitotracker stains. C: average circularity of mitochondria. D: area/perimeter ratio of mitochondria. Values not sharing the same letter are significantly different; $P < 0.05$. Values represent mean ± SE ($n = 9$). RAP, rapamycin.

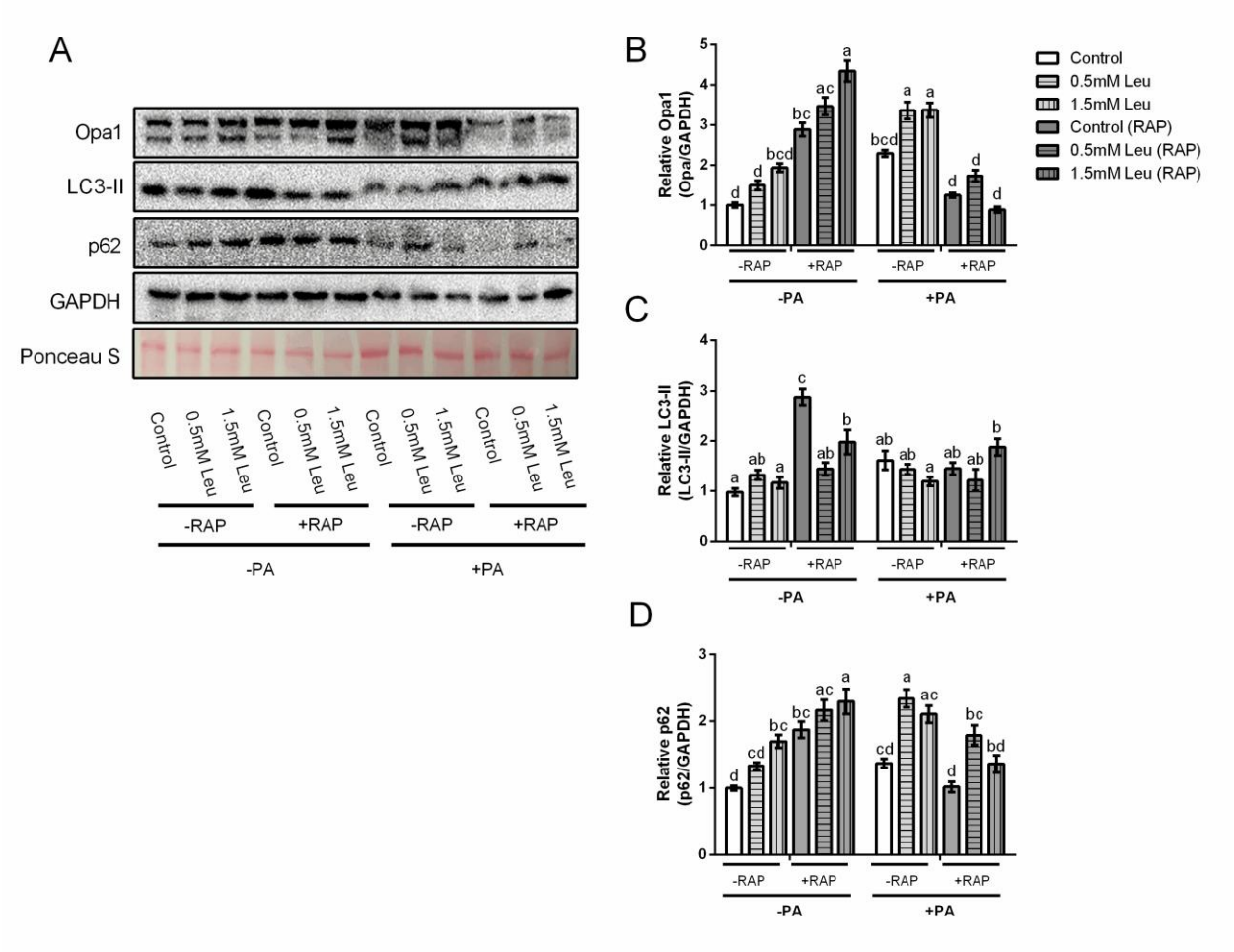

Figure 6.5. The effect of leucine (Leu) on proteins related to mitochondrial fusion and autophagy in palmitate (PA)-treated C2C12 myotubes. A: representative Western blot of mitochondrial dynamin-like GTPase (Opa1), microtubule-associated proteins 1A/1B light chain 3B (LC3-II), p62, and GAPDH. The PVDF membrane was stained by Ponceau S stain to determine the loading control. The relative protein content was expressed as the fold change to control group. B: Opa1 C: LC3-II. D: p62. Values not sharing the same letter are significantly different; $P < 0.05$. Values represent mean ± SE (n = 9). RAP, rapamycin.

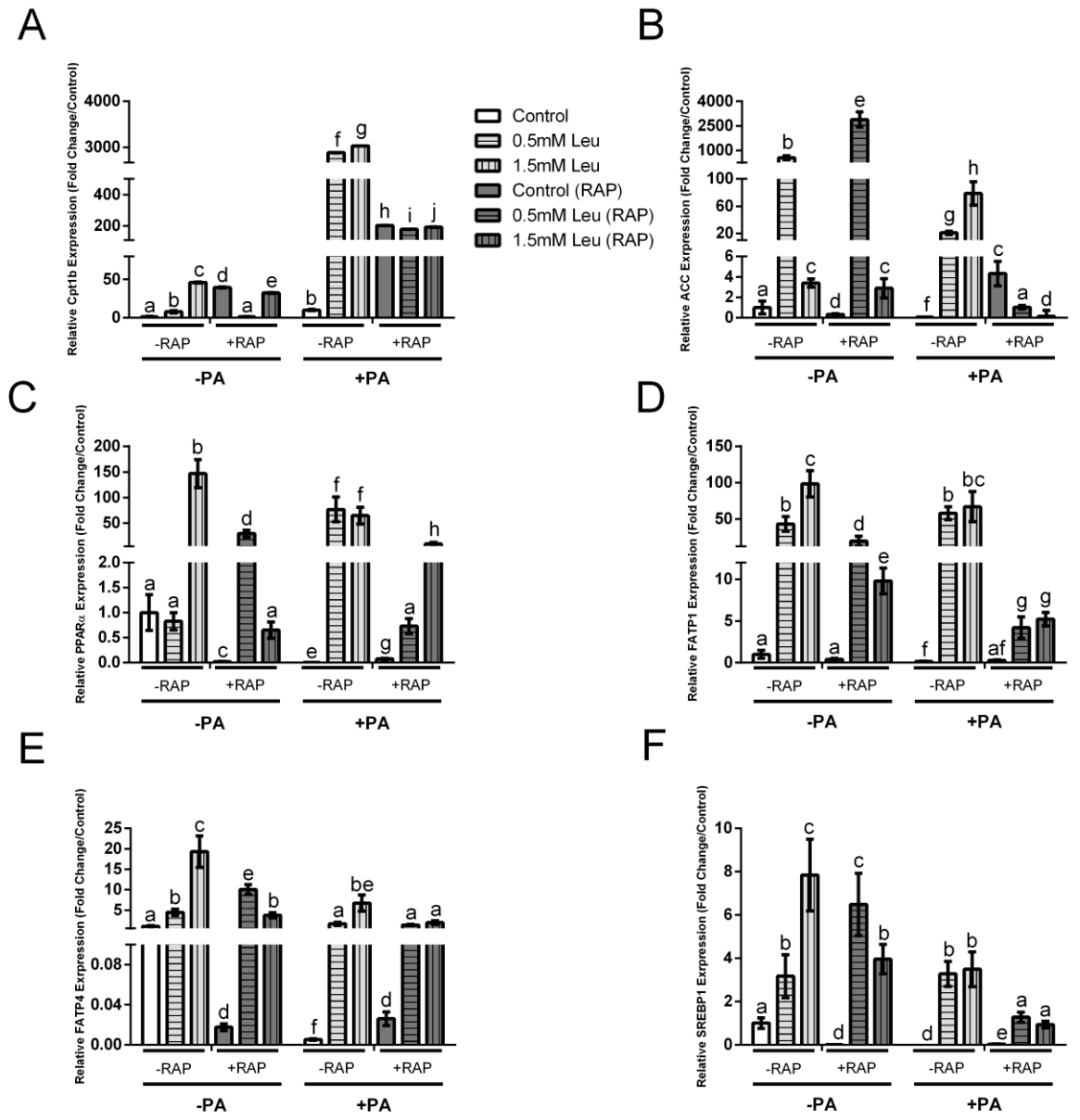

Figure 6.6. Leucine (Leu) promotes gene expression related to fatty acid metabolism in palmitate (PA)-treated C2C12 myotubes. Carnitine palmitoyltransferase (Cpt) 1b (A), acetyl-CoA carboxylase (ACC) (B), peroxisome proliferator-activated receptor α (PPARα) (C), fatty acid transport protein (FATP) 1 (D), FATP4 (E), and sterol regulatory element-binding protein 1 (SREBP1) (F). Values not sharing the same letter are significantly different; $P < 0.05$. Values represent mean ± SE (n = 9). RAP, rapamycin.

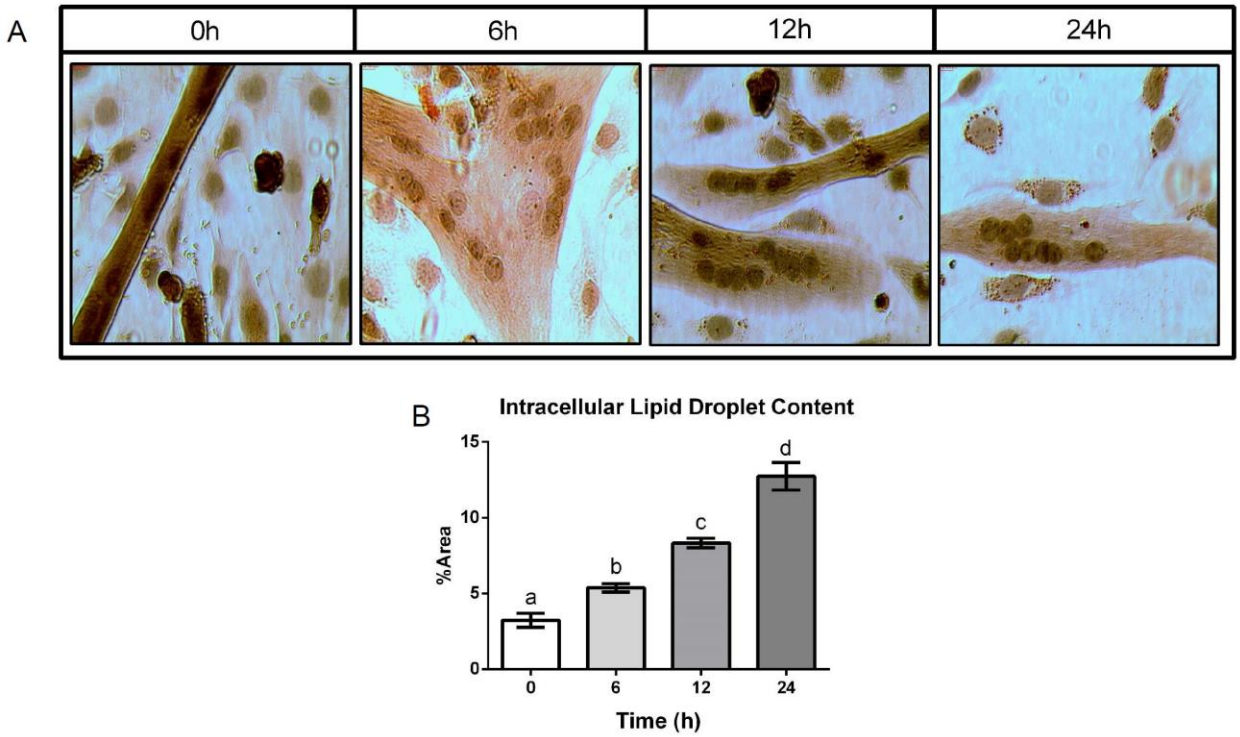

Figure 6.7. Determination of palmitate treatment time. C2C12 myotubes were differentiated from myoblasts under DMEM medium that contained 2% horse serum. Then cells were treated with 0.75mM palmitate for four periods of time: 0 hour, 6 hours, 12 hours, and 24 hours. (A) Oil red o staining of all treatments. (B) Quantification of oil red o staining of intracellular fatty acid indicates 24-hour palmitate treatment led to highest intracellular fatty acid accumulation. Different letters denote significant difference between means at level of 0.05.

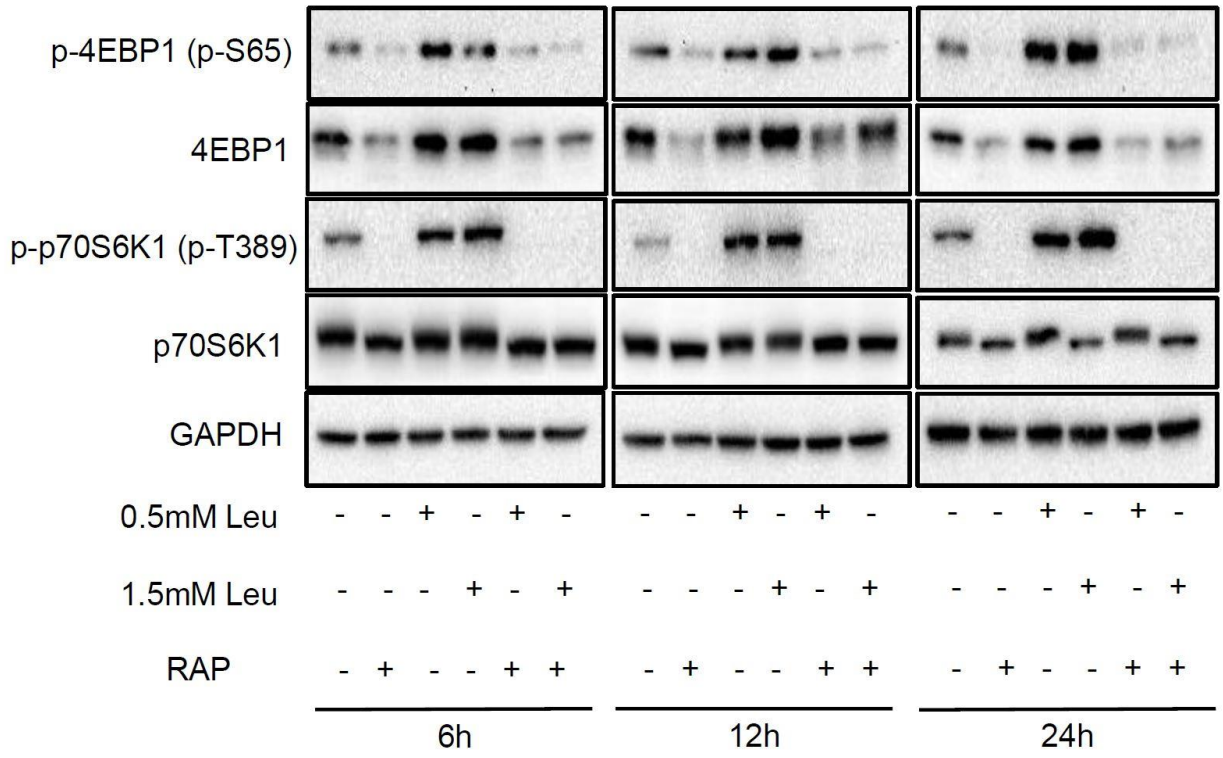

Figure 6.8. Both 0.5mM and 1.5mM leucine activate mTORC1 pathway, while inhibited by rapamycin in C2C12 myotubes. C2C12 myotubes were differentiated from myoblasts under DMEM medium that contained 2% horse serum. Then cells were treated by treatments: control, rapamycin, 0.5mM leucine, 1.5mM leucine, 0.5mM leucine + rapamycin, and 1.5mM leucine + rapamycin. Three treatment times were used for all treatment groups: 6 hours, 12 hours, and 24 hours. Western blot analysis was performed as described in the Materials and Methods to determine the phosphorylation of S65 residue of 4EBP1, total 4EBP1, phosphorylation of T389 residue of p70S6K1, total S6K1, and GAPDH.

8.1 Tables

Table 6.1. Sequences of primers used for real-time PCR

Gene Name	F/R	Sequence
Cpt1b	Forward	5'-AAGTGCGGTGATTATAGG-3'
	Reverse	5'-AAGACGGTTAAGGAGTTC-3'
ACC	Forward	5'-GATACCAGGACAGAATGAG-3'
	Reverse	5'-CAGACTTGAACCAGAACT-3'
SREBP1	Forward	5'-CAGTGTTGAGTTGTTAGAAG-3'
	Reverse	5'-CCATAAGTGTAGGCTATCC-3'
PPARα	Forward	5'-GCCAACTACATCCTATTCT-3'
	Reverse	5'-GCTCACACTCATCTCTAAT-3'
FATP1	Forward	5'-TACAGTAGGTGGTCAAGA-3'
	Reverse	5'-CTAAGGTTCACAGTCAGAT-3'
FATP4	Forward	5'-GCATTACATCGCATTATCAG-3'
	Reverse	5'-AAGAGGAGGAAGAAGAAGA-3'
ANT1	Forward	5'-AGCATGCCAGCAAGCAAAT-3'
	Reverse	5'-CGCAGTCTATGATGCCCTTGT-3'
NRF1	Forward	5'-CAGACACGTTTGCTTCGGAAA-3'
	Reverse	5'-CCCACTCGCGTCGTGTACT-3'
PGC1α	Forward	5'-CCGTAGGCCCAGGTACGA-3'
	Reverse	5'-TGCGGTATTCATCCCTCTTGA-3'
SIRT1	Forward	5'-GCCGCGGATAGGTCCATATA-3'
	Reverse	5'-TCGAGGATCGGTGCCAAT-3'
PPARγ	Forward	5'-GCCCACCAACTTCGGAATC-3'
	Reverse	5'-TGCGAGTGGTCTTCCATCAC-3'
TFAM	Forward	5'-GCACCCTGCAGAGTGTTCAA-3'
	Reverse	5'-CGCCCAGGCCTCTACCTT-3'
UCP2	Forward	5'-CCTCAAAGCAGCCTCCAGAA-3'
	Reverse	5'-CAATCGGCAAGACGAGACAGA-3'
UCP3	Forward	5'-CCACCTTAGGGCAAGAACGA-3'
	Reverse	5'-AGATGAGAAAACCTCCGAGAGAGA-3'
GAPDH	Forward	5'-GGTGAAGGTCGGAGTCAACGGA-3'
	Reverse	5'-GAGGGATCTCGCTCCTGGAAGA-3'

9 Overall conclusion

This dissertation includes studies that 1) firstly comparing gene expression related to protein breakdown and energy metabolism between young and old, lean and overweight/obese human skeletal muscle; 2) firstly linking the gene expression related protein breakdown and energy metabolism with whole body protein kinetics; 3) firstly showing leucine can decrease intramyocellular lipid deposition in an *in vivo* model of obesity independent of mTORC1 pathway. However, further *in vivo* study is needed to verify the effect of leucine on lipid deposition in skeletal muscle in obesity condition. In addition, the effect of leucine on skeletal muscle in aging condition is also required.